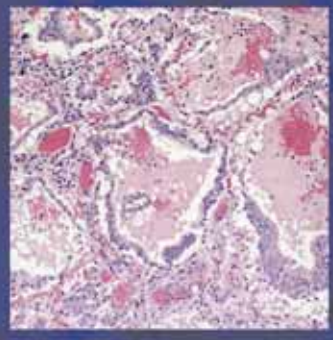
CLINICAL VIROLOGY

FOURTH EDITION



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DEDICATION

To our families—Eva, Sara, Matthew and Isabella; Kevin, Sarah, Christopher, Jennifer, and Katherine; Melissa, Dan, Gabi, Gretta, Grant, Geoff, Mary, Cotes, and Anderson—and to our many colleagues and friends, who have inspired and supported us throughout the years.

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Yuan Chang (chapter 26) holds patents that are assigned to his university on KSHV-related inventions.

Harry R. Dalton (chapter 50) has received travel and accommodation costs and consultancy fees from GlaxoSmithKline, Wantai, and Roche; travel, accommodation, and lecture fees from Merck, Gilead, and GFE Blut GmbH; and travel and accommodation fees from the Gates Foundation.

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Preface

Virology is currently one of the most dynamic areas of clinical medicine. Challenges related to novel viruses, changing epidemiologic patterns, new syndromes, unmet vaccine needs, antiviral drug resistance, and threats of bioterrorism are balanced against improved insights into viral pathogenesis, better diagnostic tools, novel immunization strategies, and an expanding array of antiviral agents. The demands on clinicians, public health workers, and laboratorians will continue to increase as will the opportunities for effective intervention. This text, now in its fourth edition, is designed to inform scientists and health care professionals about the medically relevant aspects of this rapidly evolving field.

Clinical Virology has two major sections. The first addresses infections and syndromes related to particular organ systems, as well as the fundamentals of modern medical virology, including immune responses and vaccinology, diagnostics, and antivirals. The second provides agent-specific chapters that detail the virology, epidemiology, pathogenesis, clinical manifestations, laboratory diagnosis, and prevention and treatment of important viral pathogens. In a multiauthored text like *Clinical Virology,* the selection of authors is key. The senior authors for individual chapters were chosen because of their internationally recognized expertise and active involvement in their respective fields. In addition, common templates for the syndrome-specific and separately for the agent-specific chapters allow the reader to readily access material. Since publication of the third edition in 2009, all of the chapters have been extensively revised to incorporate new information and relevant citations. The timeliness and presentation of the fourth edition have been enhanced by publication of chapters online as they have become available and by the increased numbers and incorporation of color figures into the text. New chapters on Bornaviruses and Anelloviruses have been added, and the rapidly expanding field of antiviral drugs demanded dividing the subject into four chapters.

We have been particularly fortunate in receiving invaluable help from our administrative assistants, Mayra Rodriguez, Dunia Ritchey, and Lisa Cook. In addition, we express our appreciation for the enthusiastic professional support provided by Christine Charlip, Lauren Luethy, and Larry Klein of ASM Press.

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Important Notice (Please Read)

This book is intended for qualified medical professionals who are aware that medical knowledge is constantly changing. As new information becomes available, changes in treatment, diagnostic procedures, equipment, and the use of drugs and biologicals become necessary. The editors, authors, and publisher have, as far as it possible, taken care to ensure that the information is up-to-date but cannot guarantee that it is.

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Introduction

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Clinical virology incorporates a spectrum of disciplines and information ranging from the x-ray crystallographic structure of viral proteins to the global socioeconomic impact of disease. Clinical virology is the domain of molecular biologists, geneticists, pharmacologists, microbiologists, vaccinologists, immunologists, practitioners of public health, epidemiologists, and clinicians, including both pediatric and adult health care providers. It encompasses events impacting history that range from pandemics and Jennerian vaccination to the identification of new pathogens, mechanisms of disease, and modern countermeasures like antiretrovirals. For example, since the previous edition of this text, sequencing techniques from human specimens have led to the identification of numerous new members of several virus families, including polyomaviruses, orthomyxoviruses, and bunyaviruses (1–3). New viral pathogens have emerged or been recognized, including a camel-associated coronavirus causing the SARS-like Middle East respiratory syndrome, the tick-borne zoonotic orthomyxovirus (Bourbon virus) (2), the bunyaviruses (severe fever with thrombocytopenia virus) (3) and Heartland virus (4, 5), and newly emerged avian and swine influenza viruses causing zoonotic infections (H7N9, H5N6, H6N1, H10N8, H3N2v) (6–10). A bornavirus, belonging to a virus family known to cause disease in animals but with an unproven role in human disease, has been isolated in a cluster of encephalitis cases (11). Well-recognized pathogens like Chikungunya and Zika viruses have spread geographically to cause major outbreaks in the Western Hemisphere (12, 13). The political and social consequences of vaccine denialism have delayed the eradication of polio and measles globally and resulted in re-emerging outbreaks of measles in Europe and North America. Most dramatically, the pattern of relatively limited, albeit lethal, outbreaks of Ebola virus in central Africa over the past 40 years changed in 2014 with the West African outbreak that caused over 28,000 infections leading to over 11,000 fatalities, including more than 500 health care workers, before coming under apparent control in 2016 (http://www.who.int/csr/disease/ebola/en/).

On the positive side, the development of new diagnostic technologies has provided dramatic advances for the detection of new pathogens and the diagnosis and management of virus infections in the clinic. Human "virome" projects based on high-throughput serologic screening, sequencing, and other technologies have documented the frequent but individually unique patterns of infection that we have with these microbes (14–16). Since the previous edition we have seen the revolutionary impact of combination antiviral therapy for HIV, with approximately 15 million people under treatment globally in 2015, followed by the development of 8- to 12-week interferon-free regimens for hepatitis C, with cure rates of over 95%. Modified viruses have become therapeutic tools in treating some forms of malignancy (e.g., herpes simplex virus for glioblastoma) (17, 18). In addition, promising new antiviral drugs and vaccines are in development for many other virus infections. The editors hope that the fascinating breadth and importance of the subject of clinical virology will be conveyed by this text. In this fourth edition, the editors have attempted to update and expand upon the information in the previous edition, while making the content more accessible with Internet-based technology.

A few words about nomenclature are necessary. Students (among others) are plagued by virus classification. Historically, classification reflected the information available from general descriptive biology. Viruses were thus classified by host (e.g., plant, insect, murine, avian), by disease or target organ (e.g., respiratory, hepatitis, enteric), or by vector (e.g., arboviruses). These classifications were often overlapping and inconsistent. Molecular biology now permits us to classify viruses by genetic sequence and biophysical structure, which can be quantitative and evolutionarily meaningful. Table 1, which shows the taxonomy of human viruses, is derived from the comprehensive Ninth Report of the International Committee on Taxonomy of Viruses (19).

The list in Table 1 represents viruses known to infect humans. Many of the agents are primarily animal viruses that accidentally infect humans: herpesvirus B, rabies, the Arenoviridae, the Filoviridae, the Bunyaviridae, and many arthropod-borne viruses. The role of intraspecies transmission of viruses is becoming increasingly appreciated. Although its contribution to zoonotic infections like H5N1 and antigenic shift of influenza A virus is well documented, the role of intraspecies transmission is a major consideration in the "emerging" diseases caused by Sin Nombre virus and related hantaviruses, Nipah virus, Ebola virus, arenavirus, hemorrhagic fevers, variant bovine spongiform encephalopathy, and most importantly, the human immunodeficiency viruses.

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TABLE 1 Taxonomy of human viruses

Family	Subfamily	Type species or example	Morphology	Envelope	Chapter
	Genus				
DNA viruses					
dsDNA viruses					
Poxviridae			Pleomorphic	+	19
	Chordopoxvirinae				
	Orthopoxvirus	Vaccinia virus, variola			
	Parapoxvirus	Orf virus			
	Molluscipoxvirus	Molluscum contagiosum virus			
	Yatapoxvirus	Yaba monkey tumor virus			
Herpesviridae			Icosahedral	+	
	Alphaherpesvirinae				
	Simplexvirus	Human herpesvirus 1 and 2			20
		Cercopithecine herpesvirus 1 (herpesvirus B)			21
	Varicellovirus	Human herpesvirus 3			22
	Betaherpesvirinae				
	Cytomegalovirus	Human herpesvirus 5			23
	Roseolovirus	Human herpesvirus 6 and 7			24
	Gammaherpesvirinae				
	Lymphocryptovirus	Human herpesvirus 4			25
	Rhadinovirus	Human herpesvirus 8			26
Adenoviridae	*Mastadenovirus*	Human adenoviruses	Icosahedral	–	27
Polyomaviridae	*Polyomavirus*	JC virus	Icosahedral	–	28
Papillomaviridae	*Papillomavirus*	Human papillomaviruses	Icosahedral	–	29
ssDNA viruses					
Parvoviridae			Icosahedral	–	
	Parvovirinae				30
	Erythrovirus	B19 virus			
	Dependovirus	Adeno-associated virus 2[a]			
	Bocavirus	Human bocavirus			
Anelloviridae	*Alphatorquevirus*	Torque teno virus[a]	Icosahedral	–	31
DNA and RNA reverse transcribing viruses					
Hepadnaviridae	*Orthohepadnavirus*	Hepatitis B virus	Icosahedral with envelope	+	32
Retroviridae			Spherical	+	

	Deltaretrovirus	HTLV 1 and 2			33
	Lentivirus	Human immunodeficiency viruses 1 and 2			34
	Spumavirus	Spumavirus (foamy virus)[a]			
RNA viruses					
dsRNA viruses					
Reoviridae			Icosahedral	–	
	Orthoreovirus	Reovirus 3[a]			
	Orbivirus	Kemerovo viruses			35
	Coltivirus	Colorado tick fever virus			35
	Seadornavirus	Banna virus			35
	Rotavirus	Human rotavirus			36
Negative-stranded ssRNA viruses					
Paramyxoviridae			Spherical	+	
	Paramyxovirinae				
	Respirovirus	Human parainfluenza viruses			37
	Morbillivirus	Measles virus			38
	Rubulavirus	Mumps virus			39
	Henipavirus	Nipah virus			40
	Pneumoniavirinae				
	Pneumovirus	Human respiratory syncytial virus			37
	Metapneumovirus	Human metapneumovirus			37
Rhabdoviridae			Bacilliform	+	41
	Vesiculovirus	Vesicular stomatitis virus			
	Lyssavirus	Rabies virus			
Filoviridae	*Filovirus*	Ebola virus	Bacilliform	+	42
Orthomyxoviridae			Spherical	+	43
	Influenzavirus A	Influenza A virus			
	Influenzavirus B	Influenza B virus			
	Influenzavirus C	Influenza C virus			
Bornaviridae	*Bornavirus*	Borna disease virus	Spherical	+	57
Bunyaviridae			Amorphic	+	44
	Orthobunyavirus	Bunyamwera virus, LaCrosse virus			
	Hantavirus	Hantaan virus, Sin Nombre virus			
	Nairovirus	Congo-Crimean hemorrhagic fever virus			
	Phlebovirus	Rift Valley fever virus			
Arenaviridae	*Arenavirus*	Lymphocytic choriomeningitis virus	Spherical	+	45

(*Continued on next page*)

TABLE 1 Taxonomy of human viruses (*Continued*)

Family	Subfamily	Type species or example	Morphology	Envelope	Chapter
Positive-stranded ssRNA viruses					
Picornaviridae			Icosahedral	–	
	Enterovirus	Polioviruses			46
	Rhinovirus	Human rhinoviruses			47
	Hepatovirus	Hepatitis A virus			48
Caliciviridae	*Calicivirus*	Norwalk virus	Icosahedral	–	49
Hepeviridae	*Hepevirus*	Hepatitis E virus	Icosahedral	-	50
Astroviridae	*Mamastrovirus*	Human astrovirus 1	Icosahedral	–	51
Coronaviridae	*Coronavirus*	Human coronavirus	Pleomorphic	+	52
Flaviviridae			Spherical	+	
	Flavivirus	Yellow fever virus			53
	Hepacivirus	Hepatitis C virus			54
Togaviridae			Spherical	+	
	Alphavirus	Western equine encephalitis virus			55
	Rubivirus	Rubella virus			56
Subviral agents: satellites, viroids, and agents of spongiform encephalopathies					
Subviral agents					
Satellites (single-stranded RNA)	*Deltavirus*	Hepatitis delta (D) virus	Spherical	+	58
Prion protein agents		Creutzfeld-Jakob agent	?	–	59

[a]Human virus with no recognized human disease.

Although not a documented risk, the theoretical threats of organ transplants from primates and pigs prompted a section on xenotransplantation in the chapter on transplantation. In addition, a number of human viruses have not been recognized to cause human disease, including spumaretroviruses, reoviruses, anelloviruses, and the adeno-associated parvoviruses. The text does not elaborate on these viruses in detail, but the editors did elect to include a chapter on Torque teno virus and related anelloviruses, despite any proven disease association, because of their remarkably high prevalence in human populations globally and the remarkably high titers achieved in blood. We have also added a new chapter on bornaviruses, which may represent either a newly recognized zoonosis or an emerging infection.

In order to provide a comprehensive yet concise treatment of the diverse agents and diseases associated with human viral infections, the editors have chosen to organize the textbook into two major sections. The first provides information regarding broad topics in virology, including immune responses, vaccinology, laboratory diagnosis, and principles of antiviral therapy, and detailed considerations of important organ system manifestations and syndromes caused by viral infections. The second section provides overviews of specific etiologic agents and discusses their biology, epidemiology, pathogenesis of disease causation, clinical manifestations, laboratory diagnosis, and management. We have attempted to ensure that the basic elements are covered for each of the viruses of interest, but it is the authors of each of these chapters that have done the real work and to whom we owe our gratitude and thanks.

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Section I

Viral Syndromes and General Principles

Respiratory Infections

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Respiratory viral infections have a major impact on health. Acute respiratory illnesses, largely caused by viruses, are the most common illness experienced by otherwise healthy adults and children. Data from the United States, collected in the 1992 National Health Interview Survey, suggest that such illnesses are experienced at a rate of 85.6 illnesses per 100 persons per year and account for 54% of all acute conditions exclusive of injuries (1). A total of 44% of these illnesses require medical attention and result in 287 days of restricted activity, 94.4 days lost from work, and 182 days lost from school per 100 persons per year. The morbidity of acute respiratory disease in the family setting is significant. The Tecumseh study, a family-based surveillance study of respiratory illness, estimated that approximately one-quarter of respiratory illnesses result in consultation with a physician (2). Illness rates for all acute respiratory conditions are highest in young children, and children below the age of 9 have been estimated to experience between five and nine respiratory illnesses per year (3).

Mortality due to acute viral respiratory infection in otherwise healthy individuals in economically developed countries is rare, with the exception of epidemic influenza and possibly respiratory syncytial virus. However, acute respiratory infection is a major cause of childhood mortality in low- and middle-income countries (4), and it is estimated that 4.5 million children under 5 years of age die annually from acute respiratory infection. Viruses are estimated to play a contributing role in approximately 20% to 30% of these deaths (4). In response, the World Health Organization has undertaken a major new initiative, the Battle against Respiratory Viruses (or BRaVe) to foster research on these pathogens (5).

Both RNA and DNA viruses are responsible for these infections, producing clinical syndromes ranging in severity from merely uncomfortable to life threatening. Each of these viruses may be responsible for different clinical syndromes depending on the age and immune status of the host. Furthermore, each of the respiratory syndromes associated with viral infection may be caused by a variety of specific viral pathogens (Table 1; also see Table 1 in Chapter 52). This chapter describes the clinical syndromes of respiratory virus infection, the spectrum of viruses associated with these syndromes, and the pathophysiology of these illnesses. Specific features of the virology and pathophysiology of disease induced by individual viral agents are described in greater detail in each of the virus-specific chapters.

SEASONAL PATTERNS OF RESPIRATORY VIRUS INFECTION

Many of the viruses associated with acute respiratory disease display significant seasonal variation in incidence (Fig. 1). Although the exact seasonal arrival of each virus in the community cannot be predicted with precision, certain generalizations are useful diagnostically and in planning control strategies. For example, both influenza and respiratory syncytial virus epidemics occur predominantly in the winter months, with a peak prevalence in January to March in the northern hemisphere. Although the periods of peak incidence for these two viruses usually do not coincide, there is often overlap between the two seasons. Parainfluenza virus type 3 (PIV-3) infections show a predominance in the spring, while types 1 and 2 (PIV-1 and PIV-2) cause outbreaks in the fall to early winter. Rhinoviruses may be isolated throughout the year, with increases in frequency in the spring and fall. The peak prevalence of enteroviral isolations is in late summer and early fall, while adenoviruses are isolated at roughly equal rates throughout the year. The herpesviruses do not show significant seasonal variation in incidence, except for varicella, which occurs throughout the year, but more commonly in late winter and early spring.

COMMON COLDS

Clinical Features and Syndrome Definition

Common colds are familiar to most adults and are usually self-diagnosed. Most observers consider colds to include symptoms of rhinitis with variable degrees of pharyngitis; the predominant associated symptoms include nasal stuffiness, sneezing, runny nose, and sore throat. Patients often report chills, but significant fever is unusual. Cough and hoarseness are variably present and may be more frequent in the elderly (6). Headache and mild malaise may occur. Although a multitude of viruses may be associated with this syndrome, the pattern of symptoms associated with colds does not appear to vary significantly among agents. Physical findings are nonspecific and most commonly include nasal discharge and

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TABLE 1 Estimated frequency[a] with which individual viral respiratory syndromes are caused by specific common viral pathogens

Virus	Colds	Pharyngitis	Tracheobronchitis	Croup	Bronchiolitis	Pneumonia		
						Children[b]	Adults	Immuno-compromised
RNA viruses								
Influenza virus								
Type A	+[b]	++	+++	++	+	++	++++	++
Type B	+	++	++	+	+	+	++	+
Parainfluenza virus								
Type 1	+	++	+	++++	+			
Type 2	+	++	+	++	+			
Type 3	+	++	+	+++	++	+++	+	+
Respiratory syncytial virus	++	+		++	++++	++++	++	++
Human metapneumovirus	+				++	++	+	±
Measles virus			+	+		+	+	+
Rhinovirus	++++	++	+	+	+++	++	++	+
Enterovirus	++	++			+	+	±	
Coronavirus	++	+		++	+		+	+
DNA viruses								
Adenovirus		++	+	++	++	++	++	++
Herpes simplex virus		+	±		+	+		++
Varicella virus						+	+	+
Epstein-Barr virus		++					±	+
Cytomegalovirus		+				++	±	+++

[a]The relative frequency of causation is graded semiquantitatively as follows: ±, rarely if ever reported, occasional case reports; +, causes some cases (1%-5% of cases); ++; fairly common cause, (5%-15% of cases); +++; common cause, (15%-25% of cases); ++++, major cause (>25% of cases)
[b]Individuals under the age of 5.
[c]In affected regions during outbreaks.

pharyngeal inflammation. More severe disease, with higher fever, may be seen in children.

Overall, colds are one of the most common of disease experiences. Adults average 6 to 8 colds per 1,000 person-days during the peak cold season and from 2 to 4 colds per person per year (7). Rates of colds are higher in children, who average 6 to 8 colds per year. Adults with children at home have a higher frequency of colds, and women are generally affected more often then men.

Colds are self-limited, with a median duration of illness of approximately 9 to 10 days in adults (8) and longer in children (9). Recognized complications of colds include secondary bacterial infections of the paranasal sinuses and middle ear and exacerbations of asthma, chronic bronchitis, and emphysema. Involvement of the middle ear is common, and changes in middle ear pressures have been documented following both experimentally induced as well as naturally occurring rhinovirus (10) and influenza virus (11) infection. These abnormalities are likely due to eustachian tube dysfunction and probably account for the frequency with which otitis media complicates colds. Colds are associated with symptomatic otitis media in approximately 2% of cases in adults (12) and in a higher proportion in young children (13). Rhinoviruses and other common cold viruses have been detected in middle ear fluids in approximately 20% to 40% of cases of otitis media with effusion in children (14). Infections with RSV, influenza, and adenoviruses are often also associated with otitis media (13).

Colds are also associated with detectable abnormalities of the paranasal sinuses that may or may not be evident clinically. Mucosal thickening and/or sinus exudates have been observed by computerized tomography in as many as 77% of subjects with colds (15, 16). However, clinically manifest acute sinusitis is seen only in a small (0.5% to 5%) proportion of adults with naturally occurring colds.

Clinical colds in atopic individuals may be more severe or more likely to result in wheezing than in normal individuals, and rhinoviruses have been identified as major causes of asthma exacerbations in children and adults (17). The mechanism of this increased susceptibility is unclear but may be related to an altered immune response to infection. Rhinovirus colds may increase asthma by augmenting airway allergic responses such as histamine release and eosinophil influx after antigen challenge. Rhinoviruses have also been identified as important causes of exacerbations of chronic obstructive pulmonary disease (18, 19).

Etiology and Differential Diagnostic Features

The majority of common colds are associated with infection with rhinoviruses or other picornaviruses, particularly when very sensitive techniques, such as reverse-transcriptase-polymerase chain reaction (RT-PCR), are used for diagnosis (8). Other agents frequently associated with common colds include coronaviruses and nonprimary infections with parainfluenza and respiratory syncytial viruses, with a variety of other agents implicated occasionally (Table 1).

The differential diagnosis of individuals presenting with typical signs and symptoms is limited. However, in the presence of additional signs or symptoms which are not part of this clinical description, such as high, persistent fever, signs of respiratory distress, or lower respiratory tract disease, alternative diagnoses should be sought. Allergic causes

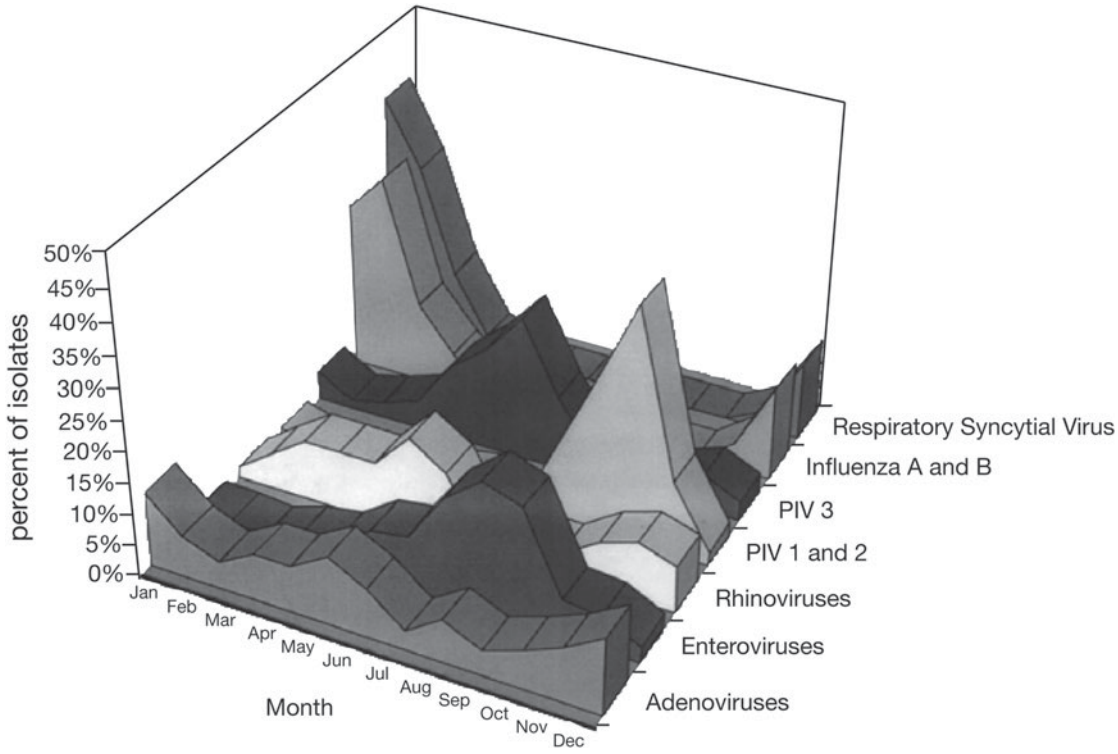

FIGURE 1 Many of the viruses that affect the respiratory tract exhibit a seasonal variation in prevalence. In this figure, numbers of virus isolates from children seen in private pediatric practices in Rochester, NY, are plotted by month of isolation. Data represent the 6-year average from 1990 to 1995 and are expressed as the percentage of all isolates of that virus that occurred in the given month.

should be considered in individuals who present with recurrent symptoms restricted to the upper respiratory tract.

Pathogenesis

Studies of the pathogenesis of the common cold have largely focused on rhinoviruses, the most commonly implicated viral etiology. Transmission of most of the viruses responsible for the common cold is by direct contact, with inoculation of virus into the upper respiratory tract. *In situ* hybridization studies of nasal biopsy specimens from rhinovirus-infected subjects demonstrate that infection is largely confined to relatively small numbers of ciliated nasal mucosal epithelial cells (20), although occasional non-ciliated cells are also infected (20). Sloughing of these epithelial cells is seen in naturally occurring colds, but the epithelial lining remains intact, with structurally normal cell borders (21). Infection is not associated with significant increases in the numbers of lymphocytes in the nasal mucosa (22), but increases in the numbers of polymorphonuclear leukocytes have been detected in nasal mucosa and secretions, probably due to elaboration of IL-8 by infected cells (23). Although rhinoviruses are not able to grow efficiently at core body temperature, virus can be detected within cells of the lower airway even in uncomplicated colds in healthy subjects (24).

In general, the number of infected cells appears to be quite limited, even in fairly symptomatic individuals (20). Such findings suggest that virus-induced cellular injury is not the direct cause of symptoms in rhinovirus colds and that inflammatory mediators and neurogenic reflexes play important roles. The nasal secretions during the initial response to rhinovirus infection are predominantly the result of increased vascular permeability, as demonstrated by elevated levels of plasma proteins in nasal secretions (25). Glandular secretions (lactoferrin, lysozyme, and secretory IgA) predominate later in colds (25). Similar observations have been made in allergic rhinitis. However, in contrast to the situation in allergic rhinitis, histamine does not appear to play a role in the induction of symptoms in colds, because nasal histamine levels do not increase, and therapy with selective (nonsedating) H1 antihistamines is not effective (26–28).

Local cytokine production is associated with symptoms in colds. Nasal secretion of kinin, IL-1, IL-6, and IL-8 levels increases during colds, and kinin and IL-8 concentrations correlate with symptoms (26). The low IL-6 production polymorphism has been associated with greater symptom magnitude following experimental rhinovirus challenge in susceptible adults (29) while polymorphisms in I-L10 or TNFα do not have a discernable effect. Intranasal administration of bradykinin mimics the induction of signs and symptoms in the common cold, including increased nasal vascular permeability, rhinitis, and sore throat (27, 30). Enhanced synthesis of proinflammatory cytokines and cell adhesion molecules in the middle ear may also contribute to the pathogenesis of otitis media associated with colds (31).

Treatment and Prevention

Treatment of colds in clinical practice is directed toward alleviation of symptoms. Symptoms of sneezing and rhinorrhea can be alleviated with nonselective antihistamines such as brompheniramine, chlorpheniramine, or clemastine fumarate, at the cost of some sedation (32, 33). The effect is probably due to the anticholinergic properties of these drugs because, as mentioned earlier, treatment with selective H1 inhibitors is not effective. Topical application of

vasoconstrictors such as phenylephrine or ephedrine provides temporary relief of nasal obstruction but may be associated with a rebound of symptoms upon discontinuation if used for more than a few days. Studies of pseudoephedrine have demonstrated measurable improvements in nasal air flow consistent with a decongestant effect (34, 35). Nonsteroidal anti-inflammatory drugs such as naproxen moderate the systemic symptoms of rhinovirus infection (36). Symptomatic therapy with systemic anticholinergic drugs or anticholinergic-sympathomimetic combinations has not been shown to confer any benefit and to be associated with significant side effects (37). In particular, the use of the decongestant phenylproponolamine has been shown to be associated with an increased risk of hemorrhagic stroke (38, 39), and this drug has been removed from over-the-counter cold remedies. However, topical application of ipratropium, a quaternary anticholinergic agent that is minimally absorbed across biologic membranes, reduces rhinorrhea significantly in naturally occurring colds (40). This agent probably exerts its major effect on the parasympathetic regulation of mucous and seromucous glands.

As expected, there is no benefit in treatment of colds with antibacterial agents, although they are frequently prescribed in colds, particularly in children. Echinacea has been suggested as having efficacy in colds, but a recent randomized trial showed no benefit (41), and administration of this remedy is not associated with a shorter duration of symptoms (42). Zinc gluconate may slightly reduce the duration of colds but does not reduce symptom severity and is associated with a high frequency of adverse events (43).

Clinical Features and Syndrome Definition

Pharyngitis is a common complaint of both adults and children and is one of the more common reasons for seeking outpatient medical care. In general, this syndrome refers to individuals who present with the primary complaint of sore throat and should probably be reserved for those individuals who manifest some objective evidence of pharyngeal inflammation as well. The clinical manifestations of pharyngitis are dominated by the specific causative agent and can be divided into those cases in which nasal symptoms accompany pharyngitis, which are predominantly viral in nature, and those cases without nasal symptoms, which have a somewhat more diverse spectrum of etiologic considerations, including both group A and nongroup A streptococci, chlamydia (strain TWAR), mycoplasma, and other agents (44).

Etiology and Differential Diagnostic Features

Viral pathogens associated with acute pharyngitis are summarized in Table 1. Rhinovirus colds are frequently accompanied by pharyngitis, although objective signs of pharyngeal inflammation are uncommon. Adenovirus infections are frequently associated with pharyngitis, and a specific syndrome of pharyngoconjunctival fever, consisting of fever, pharyngitis, and bilateral conjunctivitis is associated with adenovirus types 3 and 7. A variety of enteroviral serotypes are associated with febrile pharyngitis. Herpangina is a specific coxsackievirus-induced pharyngitis in which small (1 to 2 mm) vesicular lesions of the soft palate rupture to become small white ulcers. Pharyngitis is a typical component of acute influenza in which individuals experience the sudden onset of systemic symptoms of fever, myalgias, and malaise accompanied by upper respiratory signs and symptoms including pharyngitis. Primary oral infection with herpes simplex virus may present with pharyngitis, typically with vesicles and shallow ulcers of the palate, and cervical lymphadenopathy.

Pharyngitis will be a significant complaint in approximately one-half of cases of the acute mononucleosis syndrome due to Epstein-Barr virus (45). Pharyngitis in this syndrome is generally exudative and is accompanied by cervical and generalized lymphadenopathy, as well as fever, hepatosplenomegaly, and other systemic symptoms. The heterophile antibody test is typically positive in the second week of illness. Cytomegalovirus can cause an identical syndrome that is monospot-negative and may be associated with pharyngitis more commonly in children than in adults. An acute mononucleosis-like syndrome with pharyngitis may also be the presenting manifestation of primary HIV infection. Viruses associated with hemorrhagic fever, such as Ebola, Marburg, or Lassa, produce an acute pharyngitis that occurs early in the disease, before skin lesions appear.

The differential diagnosis of acute pharyngitis generally centers upon the differentiation of streptococcal from viral etiologies. Features suggestive of streptococcal pharyngitis include tonsillar swelling, moderate to severe tenderness on palpation, enlargement of lymph nodes, presence of scarlatiniform rash, and absence of coryza (46). The bacterium *Fusobacter necrophorum* has also been recognized as frequently associated with acute pharyngitis in adults and has a clinical presentation similar to that of streptococcal pharyngitis (47).

The presence of nasal symptoms or of conjunctivitis favors a viral etiology, and as described above, some viral syndromes may present with distinguishing characteristics that help in their identification. Generally, acute pharyngitis in children less than 3 years of age is predominantly viral in origin. The presence of exudate is suggestive of bacterial etiology, but exudates may also be seen with adenovirus or EBV. Rapid diagnostic tests for the office identification of group A streptococci are widely available and are indicated in most cases where the etiology is uncertain. When highly sensitive tests are used, backup cultures are generally not necessary (48).

Pathogenesis

The pathophysiology of those virus infections for which pharyngitis is part of the clinical presentation is described in the individual virus-specific chapters of this book. As described above, pharyngitis in the common cold is probably the result of chemical mediators of inflammation, which are potent stimulators of pain nerve endings. Potentially similar mechanisms may account for pharyngitis in other viral syndromes as well. Direct viral damage and other host inflammatory responses may also contribute.

Treatment and Prevention

The treatment of most cases of viral pharyngitis is symptomatic, as noted in the section on common colds. Patients suspected of having influenzal pharyngitis who are seen within the first 2 days of illness can be treated with antiviral therapy (see Chapters 14 and 43). In immunosuppressed patients with chronic herpetic pharyngitis or normal hosts with primary gingivostomatitis, acyclovir therapy is recommended (see the discussion on herpes simplex virus).

Treatment of group A streptococcal infections with antimicrobial agents is generally initiated to prevent rheumatologic complications of this infection and is associated with more rapid resolution of symptoms, although the absolute benefits are rather modest (49). Rapid diagnostic tests are widely available for the office identification of group A

streptococci and are indicated in most cases where the etiology is uncertain. Antibiotic treatment based on only positive rapid test or throat culture results can reduce unnecessary use of antibiotics for treatment of pharyngitis (50).

CROUP (ACUTE LARYNGOTRACHEOBRONCHITIS)

Clinical Features and Syndrome Definition

Croup, or viral laryngotracheobronchitis, is a clinically distinct illness that predominantly affects children under the age of three. The illness typically begins with upper respiratory tract symptoms of rhinorrhea and sore throat, often with a mild cough. After 2 or 3 days, the cough deepens and develops a characteristic brassy, barking quality, which is similar to a seal's bark. Fever is usually present, generally between 38° and 40°C, although those with croup due to respiratory syncytial virus may have normal temperatures. The child may appear apprehensive and most comfortable sitting forward in bed. The respiratory rate is elevated but usually not over 50; this contrasts with bronchiolitis, in which more severe tachypnea is often seen. Chest wall retractions, particularly in the supraclavicular and suprasternal areas, may be observed. Children with this finding on presentation have a higher risk of hospitalization or of requiring ventilatory support.

The characteristic physical finding of croup is inspiratory stridor. Inspiration is prolonged, and in very severe cases, some degree of expiratory obstruction may also be seen. Rales, rhonchi, and wheezing, which reflect the characteristic involvement of the lower respiratory tract, may be heard on physical examination. A fluctuating course is typical, and the child may appear to worsen or improve within an hour. Hypoxemia occurs in 80% of children with croup severe enough to require hospitalization. The degree of hypoxia is generally difficult to ascertain clinically, but continuous monitoring of pulse oximetry does not correlate with respiratory distress and may lead to increased hospitalization rates.

Children who develop respiratory insufficiency as a result of increasing fatigue also may have elevations in $PaCO_2$. Other routine laboratory assays are generally unremarkable. Children with croup characteristically exhibit subglottic narrowing of the tracheal air shadow on PA films of the neck, the so-called "steeple" sign (Fig. 2). This finding may be useful in differentiating croup from epiglottitis. Chest X-rays may reveal parenchymal infiltrates which are part of the characteristic involvement of the lower respiratory tract in this syndrome.

Croup is predominantly a disease of young children, with a peak age incidence in the second year of life. In the Seattle virus watch family study, the annual incidence of croup was 5.2 per 1,000 in the first 6 months of life, 11.0 per 1,000 in the second 6 months, 14.9 per 1,000 in the second year of life, and 7.5 per 1,000 in those 2 to 3 years of age, with a marked drop after that age (51). Boys are somewhat more likely to be affected than girls (52).

Etiology and Differential Diagnostic Features

Overall, viruses are recovered from croup cases more frequently than from other types of respiratory illnesses. An estimate of the relative importance of individual infectious agents in croup is shown in Table 1. The parainfluenza viruses are the most common viruses responsible for croup, accounting for about 75% of cases (52). Of the parainfluenza viruses, types 1 and 2 are most commonly associated with croup (52), and the seasonal incidence of croup reflects the seasonal variations in parainfluenza virus incidence (Fig. 1). Less common causes of croup include respiratory syncytial virus, influenza A or B viruses, rhinoviruses, and adenoviruses, as well as *Mycoplasma pneumoniae*. Recent studies have also shown a strong association with the novel coronavirus NL63 (53). Measles is a relatively less common cause of croup but is associated with especially severe disease (54). Mycoplasma pneumoniae and influenza viruses tend to be isolated more commonly from older children with croup (52). Parainfluenza virus type 2 and influenza A viruses are associated with more severe disease (55), but generally the clinical presentation of the croup syndrome due to individual agents is similar. Specific viral diagnosis is not routinely performed since the clinical syndrome is sufficient for diagnosis, and management generally does not depend on identification of the specific agent.

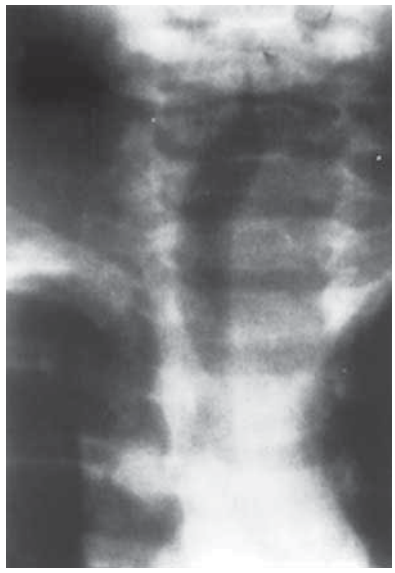

FIGURE 2 Posteroanterior roentgenogram of the neck of a child with viral croup that shows the characteristic narrowing of the air shadow of the trachea in the subglottic area. (Courtesy of Dr. Carolyn B. Hall, University of Rochester)

The majority of cases of inspiratory stridor in children are caused by viral croup. However, it is critical to distinguish these syndromes from other, potentially more serious causes of airway obstruction such as bacterial epiglottitis and tracheitis early in clinical management. Epiglottitis is an acute cellulitis of the epiglottis and surrounding structures. Patients present with acute respiratory distress and drooling, but the barking cough of croup is absent. Epiglottitis in children is usually caused by *Hemophilus influenzae* type b (Hib). The incidence of invasive Hib infections has declined remarkably since the introduction of polysaccharide-conjugate vaccines, and the incidence of epiglottitis in children has also declined considerably (56). In adults, and rarely in children, epiglottitis may be caused by a variety of other bacterial agents such as *Haemophilus parainfluenzae* or β-hemolytic streptococci, which may spread from a contiguous focus of infection. Bacterial tracheitis is a relatively rare syndrome that mimics croup. Abundant purulent sputum is often present. Bacterial tracheitis is usually caused by *Staphylococcus aureus* or Hib; other bacteria such as β-hemolytic streptococci and *Streptococcus pneumoniae* have also been associated with this syndrome. Other infectious causes of stridor, including peritonsillar or retropharyngeal

FIGURE 3 Lateral neck films of the neck in a child with epiglottitis demonstrates the characteristic thickening of the epiglottis in this disease and may be helpful in distinguishing this illness from croup or retropharyngeal abscess. (Courtesy of Dr. Caren B. Hall, University of Rochester)

abscess or diphtheria, and noninfectious causes of stridor such as trauma or aspiration of a foreign body, should be considered.

Direct visualization of the epiglottis may be necessary to exclude bacterial etiologies, and facilities and personnel for this procedure and for emergency airway management should be available. Lateral neck radiographs may show edema of the epiglottis in epiglottitis (Fig. 3) or thickening of the retropharyngeal space in individuals with retropharyngeal abscess. However, radiographs are limited in accuracy and should be performed with caution in individuals with respiratory distress. It may be useful to administer racemic epinephrine, because a rapid response is suggestive of croup.

Pathogenesis

The severity of clinical symptoms in viral croup appears to be directly related to the level of virus replication (57). This results in inflammation in both the upper respiratory tract and the lung parenchyma. The classic signs of croup, including the barking cough and inspiratory stridor, arise mostly from inflammation occurring in the larynx and trachea. Inflammatory changes are seen by histology in the epithelial mucosa and submucosa of the larynx and trachea. The cellular infiltrate includes histiocytes, lymphocytes, plasma cells, and polymorphonuclear leukocytes. The inflammation and obstruction are greatest at the subglottic level, which is the least distensible part of the airway because it is encircled by the cricoid cartilage. Consequently, localized inflammation and edema lead to obstruction of airflow. The impeded flow of air through this narrowed area produces the classic high-pitched vibration. Obstruction is greater during inspiration because the narrowing occurs in the extrathoracic portion of the airway and is enhanced in small children because the walls of the airways in these individuals are relatively compliant and can collapse to a greater extent. Obstruction of airflow results in an initial decline in tidal volume, which is compensated by an increase in respiratory rate to maintain adequate alveolar ventilation. However, if the obstruction increases, the work of breathing may increase until the child tires, and as the respiratory rate declines, the child develops hypercarbia and respiratory failure.

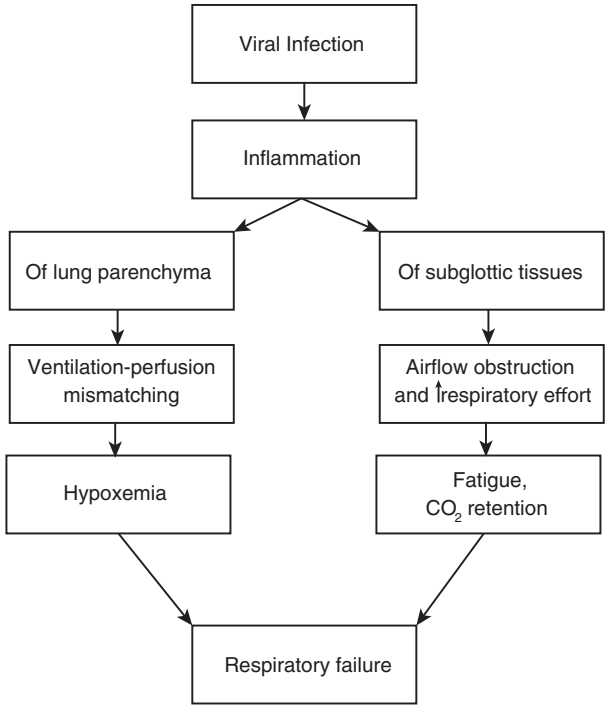

FIGURE 4 Pathophysiology of croup. Both mechanical obstruction of airflow and ventilation-perfusion mismatching due to parenchymal infection of the lung are responsible for the hypoxia and respiratory distress of croup. (Modified from Hall, Reference 206, with permission)

Involvement of the lower respiratory tract is integral to the pathophysiology of croup (Fig. 4) (58). Inflammatory changes are noted throughout the respiratory tract, including the linings of the bronchi, bronchioles, and even the alveoli. Consistent with these findings, hypoxemia is detected in about 80% of children hospitalized with croup. Although some degree of hypoxia can be explained on the basis of hypercarbia, the major pathophysiologic mechanism is ventilation-perfusion mismatching. Pulmonary edema may complicate severe croup and upper airway obstruction (59). The onset of pulmonary edema often occurs immediately following intubation. Pulmonary edema in these cases does not appear to be due to pulmonary artery hypertension but to local hypoxia and increased alveolar-capillary transmural pressure.

Treatment and Prevention

Because the majority of hospitalized children are hypoxic, oxygen is the mainstay of treatment for severe disease and should be given to all hypoxemic patients. Humidified air or mist therapy is commonly used and has several potential roles. Desiccation of the inflamed epithelial surfaces is decreased, and the viscosity of the exudate is reduced. However, the value of mist therapy has not been proven, and removal of the child from the parents and placement in a mist tent can be more distressing to the child than beneficial.

Corticosteroids have been shown to confer significant benefits in the management of mild, moderate, and severe croup, including more rapid improvement in symptoms, reduced length of hospital stay, and reduced rates of intubation. Administration of a single dose of 0.6 mg/kg dexamethasone

intramuscularly (60), an oral dose of between 0.6 to 0.15 mg/kg orally (61), or of 2 mg of budesonide by nebulizer (62) are all effective, and comparative trials have shown all three strategies to be equally effective (63, 64). Administration of single-dose corticosteroid therapy in this setting has not been associated with significant side effects and should probably be used in most patients with significant illness (65).

Administration of nebulized racemic epinephrine generally gives rapid, symptomatic relief in croup (66). It is believed that α-adrenergic stimulation by this drug causes mucosal vasoconstriction, leading to decreased subglottic edema. Several randomized trials have demonstrated a rapid beneficial effect on airway obstruction (67, 68). The onset of action is rapid, often within minutes, but the duration of relief is also limited, lasting 2 hours or less. Therefore, treated subjects should be observed closely for clinical deterioration. While symptomatic relief is considerable, use of epinephrine is not associated with improvements in oxygenation, probably because the defect in oxygen is associated with ventilation-perfusion mismatching due to lower respiratory tract involvement. In addition tachycardia may occur. Thus, inhaled epinephrine is generally reserved for children who fail to respond to more conservative management (69). Oxygen mixed with helium (heliox) has been suggested as an intervention to reduce the work of breathing; however, its role in the routine management of croup remains undetermined (70).

Antiviral agents effective against some of the viruses responsible for croup are available but have not been tested for efficacy in this situation. However, the potential benefit of the use of antiviral agents in the typical self-limited course of croup would likely be limited. Since croup is a viral illness, antibiotic therapy is of no benefit.

BRONCHIOLITIS

Clinical Features and Syndrome Definition

Bronchiolitis is a characteristic syndrome of infants whose presenting symptoms are dominated by the major pathophysiologic defect, obstruction to expiratory airflow (71). The onset of lower respiratory symptoms is usually preceded by rhinitis, often with nasal congestion and discharge. More severe symptoms characteristically occur 2 to 3 days later but in some cases are concurrent with the onset of upper respiratory symptoms. In many instances, there may be a history of exposure to an adult or sibling with a cold or other minor respiratory illness or history of exposure to other cases of bronchiolitis in the daycare setting.

The hallmark of disease is wheezing, which can be quite marked, with flaring of the nostrils and use of accessory muscles of respiration. Cough may or may not be prominent initially, and when cough is present, it may be paroxysmal in nature. Slight cyanosis is often observed, but the presence or absence of cyanosis is not a reliable indicator of the degree of oxygenation or of the severity of disease. Physical findings are generally confined to the chest, with development of rales, which are usually musical in the beginning and then become more moist. Hyper-resonance of the chest may be observed, and the liver may be displaced downward due to hyperinflation. The respiratory rate is elevated, with rates of 50 to 80 breaths per minute. Fever is frequently present at the beginning of the illness but may no longer be present at the time lower respiratory tract involvement develops. Among hospitalized infants, one-third or more are afebrile, despite marked lower respiratory tract disease. Thus, the presence or absence of fever does not indicate the severity of the child's illness. Mild conjunctivitis is noted in about a third of cases, with pharyngitis of varied severity in about half, and otitis media in 5% to 10%. The hospital course is variable, but most infants will show improvement in 3 to 4 days (72).

Radiologic findings are generally nonspecific, with reported findings including air trapping, consolidation, and collapse (73). Air trapping is particularly indicative of respiratory syncytial virus (RSV)-associated bronchiolitis and may be the only radiologic finding (Fig. 5). However, there is no correlation between the radiographic findings and the clinical course (74). Chest radiographs should be obtained to rule out alveolar filling defects suggestive of bacterial pneumonia and in those infants with severe disease, sudden deterioration, or underlying disorders (75). Results of routine laboratory tests are generally unremarkable, and the peripheral white blood cell count is usually not elevated. Abnormal water, electrolyte, and endocrine homeostasis may be seen during acute illness, including elevated antidiuretic hormone secretion and low fractional excretion of sodium (76). Electrolyte disturbances, most notably hyponatremia, may be seen with severe disease, particularly if excessive amounts of hypotonic fluid are administered (77). Acute disease may be associated with elevations in pulmonary artery pressure, but echocardiographic studies are usually unremarkable in infants with structurally normal hearts (78).

Bronchiolitis is a disease predominantly of infancy, and the epidemiology of this disease closely parallels that of the major infectious cause, respiratory syncytial virus. The peak age incidence is between 2 and 6 months of age, with over 80% of cases occurring in the first year of life (79). The risk of hospitalization of infants during the first 12 months of life for bronchiolitis has been estimated to be approximately 10 per 1,000 population (80), with the peak age of hospitalization between 1 and 3 months. Hospitalization rates are highest in children who reside in industrialized urban settings (81). Among lower socioeconomic status groups, bronchiolitis hospitalization rates of 0.5% to 1% of the entire population of infants in the first year of life are not uncommon (82).

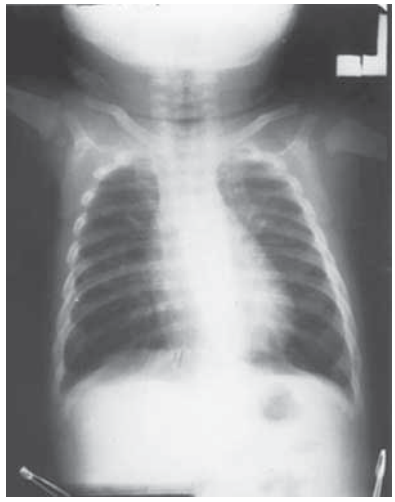

FIGURE 5 The CXR in bronchiolitis characteristically shows hyperinflation due to obstruction to airflow. A variety of other findings may be present, including interstitial infiltrates or lobar consolidation. (Courtesy of Dr. Caren B. Hall, University of Rochester)

The risk of hospitalization and severe bronchiolitis is particularly high in infants with congenital heart or lung disease or immunodeficiency (83, 84). In addition, infants born prematurely and those who are less than 6 weeks of age at the time of presentation are also at risk (85). More severe disease has also been documented in children with a family history of asthma (85) and those exposed to cigarette smoke in the family setting (86).

Etiology and Differential Diagnostic Features

The spectrum of viruses associated with bronchiolitis is shown in Table 1. RSV causes the majority of cases of bronchiolitis, and during the RSV epidemic season, essentially all cases are due to this virus (87). Overall, RSV is recovered from about three-fourths of all infants admitted to the hospital with bronchiolitis (71). Children hospitalized with bronchiolitis due to RSV tend to be younger than those with other viruses (88). Children with a higher viral load on nasopharyngeal aspirates have a higher risk of ICU admission (89). Human metapneumovirus (hMPV) is also a significant cause of bronchiolitis (90–92). The clinical picture most closely resembles that of RSV, and bronchiolitis is the major manifestation in children. Clinical features include wheezing and hypoxia. There are no clinical features that can distinguish between disease caused by hMPV and RSV, although generally RSV may be more severe.

Rhinoviruses have recently been recognized as associated with a significant proportion of cases of bronchiolitis and represent the second most common virus detected using sensitive nucleic acid tests in children with bronchiolitis. The true attribution of RV to this syndrome must take into account the frequent detection of this virus in asymptomatic children as well. Rhinoviruses can also mimic RSV infection in infants with bronchopulmonary dysplasia (93).

Other respiratory viruses causing bronchiolitis include parainfluenza viruses, influenza virus, mumps, and rhinoviruses. Adenoviruses types 3, 7, and 21 are relatively uncommon causes but may be associated with more severe disease, including bronchiolitis obliterans (94). Novel human coronaviruses, such as NL-63, have also been associated with lower respiratory tract disease in infants (95). An additional recently described human parvovirus, the human bocavirus, has been found in as many as 12% of cases of acute wheezing in young children (96).

The differential diagnosis of diseases characterized by expiratory airflow obstruction in infants is relatively small. Pertussis can occasionally be confused with bronchiolitis; more frequent vomiting, more paroxysmal cough, and lymphocytosis are clues to the diagnosis. Differentiation of acute infectious bronchiolitis from the initial presentation of allergic asthma is difficult and contributes to the difficulty in assessing therapeutic interventions in this disease. Anatomic defects such as vascular rings can cause obstruction of the airway. Foreign bodies should be considered strongly, especially in young infants. Gastroesophageal reflux is an additional consideration.

RSV and some of the other viral agents responsible for bronchiolitis can be isolated from nasopharyngeal secretions in cell culture, but nucleic acid detection techniques are more sensitive and detect a wider range of viruses (97). Rapid antigen detection techniques are widely used, but the sensitivity of such techniques is dependent on the quality of the nasopharyngeal specimen, with nasopharyngeal aspirates superior to brushings or swabs (98). Their utility in routine management is unclear, although they may be useful for infection control purposes.

Pathogenesis

The pathophysiology of infectious bronchiolitis has been described most completely in the case of infection with RSV. The basic pathophysiologic changes in bronchiolitis are summarized in Figure 6 (71). Viral infection of epithelial cells of the bronchioles leads to destruction and necrosis of the ciliated epithelium. Leucocytes, predominantly lymphocytes, can be seen in increased numbers in the peribronchial tissues (99). The submucosa becomes edematous, and there is increased production of mucus. Ultimately, dense plugs of alveolar debris and strands of fibrin form within small bronchi and bronchioles, which may partially or completely obstruct airflow. The pathogenic basis for respiratory difficulty in bronchiolitis is related to obstruction of these small airways (71). Hypoxemia is the major abnormality of gas exchange, with ventilation-perfusion imbalance the major cause of the hypoxemia. In addition to hypoxia, hypercarbia, and respiratory acidosis have been observed in some severely ill infants.

Infants appear to be particularly susceptible to the consequences of viral infection because the peripheral airways are disproportionately narrow in the early years of life. In addition, collateral channels of ventilation, such as the pores of Kohn, are deficient both in number and size in the infant lung. Finally, the airways of infants are intrinsically more reactive to bronchospastic stimuli than are the airways of older children (100). It is not clear how RSV infection results in the observed histologic damage, and the reasons some children experience relatively mild disease while others go on to respiratory failure are unknown.

The possibility that immune responses are involved in the pathogenesis of RSV bronchiolitis has received considerable attention. Factors identified as potentially playing a role include overproduction of IgE in response to infection, alteration in the cytokine phenotype of responding T cells, and release of leukotrienes in the airways (101). In addition, neural mechanisms of airway smooth muscle tone may be disrupted by RSV (102).

The innate immune response also plays an important role in the pathogenesis of RSV disease in infants, and it has been recognized that single nucleotide polymorphisms in several genes that control the inflammatory response have an important impact on the severity of RSV disease. Examples include polymorphisms in the genes for IL-4, IL-8, and IL-13, and in TLR-4 and the CCR5 receptor, among others (103).

Following recovery from acute bronchiolitis, some children experience continued episodes of wheezing, especially during apparently viral upper respiratory infections. Estimates are that the risk of either infrequent or frequent wheezing following recovery from documented RSV lower respiratory tract infection is increased by about 3- to 4-fold (104). The risk of subsequent wheezing is also increased in children with bronchiolitis associated with RSV. The mechanisms underlying this increased risk are unknown. Other studies have shown no difference in the rate of subsequent asthma in monozygotic twins discordant for RSV hospitalization (105). A history of maternal asthma may be associated with more severe disease in children with rhinovirus-associated bronchiolitis but not RSV (106, 107).

Treatment and Prevention

Recommendations regarding the treatment and prophylaxis of bronchiolitis have been summarized recently (108). Correction of hypoxemia is the most important aspect of

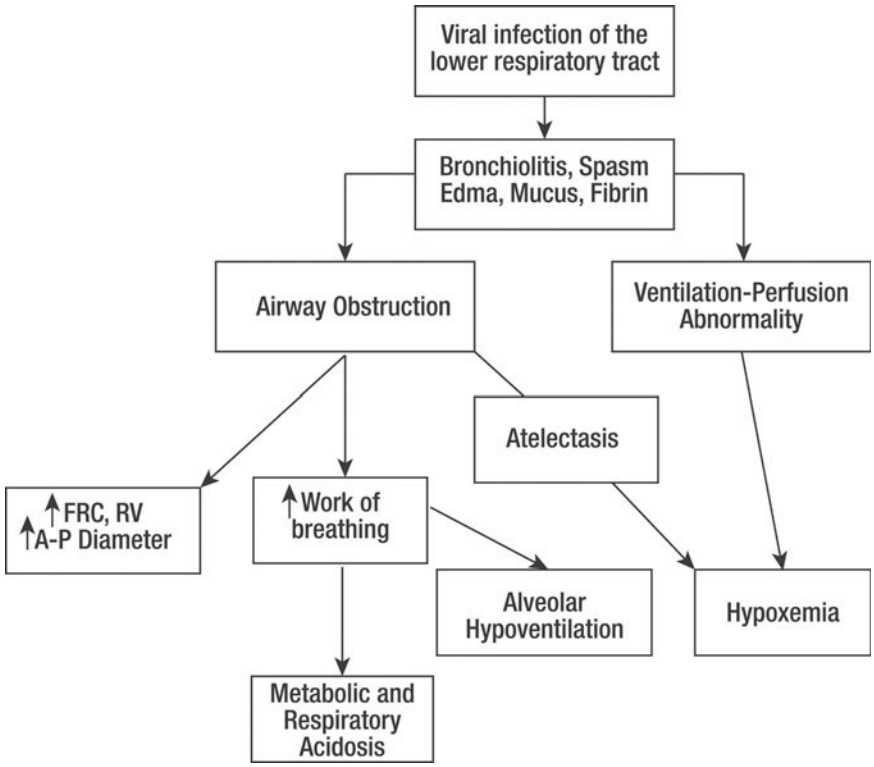

FIGURE 6 Pathophysiology of bronchiolitis. Viral infection of the lower respiratory tract results in inflammation and increased mucus production. Both airway obstruction and ventilation-perfusion mismatching are responsible for the clinical findings of hypoxia, hyperinflation, and hypoventilation. If uncorrected, these defects can lead to apnea or sudden death. (Modified from Wohl and Chernick, Reference 71, with permission)

managing RSV lower respiratory tract disease. Oxygen should be administered to infants whose saturation consistently falls below 90%, but the role of continuous monitoring of oxygen saturation is controversial. Inhaled hypertonic saline has been suggested as a modality to rehydrate the airway and may reduce the risk of hospitalization, although not affecting length of stay (109). Some studies have suggested that a humidified high-flow nasal cannula or continuous positive airway pressure may be useful in children who are at risk for respiratory failure (110).

Because of the dehydrating effect of tachypnea and reduced oral intake in some hospitalized infants, parenteral rehydration is often needed, but care must be taken to avoid inducing hyponatremia. Fluid intake and electrolyte concentrations should be carefully monitored in all infants with severe bronchiolitis, because hyponatremia and syndrome of inappropriate secretion of antidiuretic hormone (SIADH) may occur.

Other therapies are generally not routinely recommended in the treatment of bronchiolitis. Generally, bronchodilators produce modest short-term improvements in clinical scores but do not improve oxygenation, rates of hospitalization, or duration of hospital stay (111). The majority of studies of systemic corticosteroids have also failed to demonstrate a beneficial effect in acute bronchiolitis, and oral corticosteroids do not appear to have beneficial effects (112). Antibacterial drugs, including azithromycin, are of no benefit (113).

A humanized neutralizing monoclonal antibody to the RSV F protein, palivizumab (Synagis®), has had significant protective efficacy in a population of infants with prematurity or bronchopulmonary dysplasia, as well as in children with hemodynamically significant congenital heart disease. Administration of palivizumab intramuscularly at a dose of 0.15 mg/kg of body weight once per month resulted in a 55% reduction in RSV-related hospitalizations and a lower incidence of intensive care unit admissions in this population (114). Recommendations for the use of passive antibody prophylaxis in the United States have been recently revised (115). Palivizumab should generally be used only in the first year of life during the RSV epidemic season. Use is recommended in preterm infants who were born before 29 weeks' gestation (who would be expected to receive little placental transfer of maternal antibody), preterm infants of any gestational age who develop chronic lung disease of prematurity, and infants with hemodynamically significant chronic heart disease. Use can also be considered during the first year of life in infants with anatomic pulmonary disorders or neuromuscular disorders that impair clearing of secretions. The risk of severe RSV in the second year of life is considerably less, but use of palivizumab can be considered in infants with chronic lung disease of prematurity who continue to require medical support, and infants who are profoundly immunocompromised. Routine use in children with cystic fibrosis is not currently recommended.

Interruption of nosocomial transmission may be facilitated by thorough handwashing, decontamination of surfaces and inanimate objects, and isolation or cohorting of infected infants. Use of disposable eye-nose goggles by pediatric staff reduces the risk of nosocomial RSV infection in both staff and patients. Regular use of gowns, gloves, and possibly masks by hospital staff caring for infected children

may also reduce the risk of nosocomial RSV spread. Protective isolation of high-risk infants or deferring their elective admission has been recommended during institutional outbreaks of RSV.

Vaccines are available to prevent bronchiolitis due to influenza virus and mumps, but there is no vaccine currently available for prevention of bronchiolitis due to RSV or PIV. There are multiple significant hurdles to the development of such vaccines, including the very young age at which the disease presents, the suppressive effect of maternal antibody on vaccine responses, and in the case of RSV, the potential for enhanced disease in vaccine recipients (116).

TRACHEITIS AND TRACHEOBRONCHITIS

Clinical Features and Syndrome Definition

In addition to causing croup and bronchiolitis, viral infection of the trachea and bronchi may cause tracheitis or tracheobronchitis. Tracheitis is characterized by tracheal tenderness, which can be elicited by gentle pressure on the anterior trachea just below the cricoid cartilage. Substernal discomfort on inhalation, and nonproductive paroxysmal cough are noted. Paroxysmal nonproductive cough is also characteristic of tracheobronchitis and is usually much more severe at night. Later in the course of illness, small amounts of clear or whitish sputum may be produced. Accompanying symptoms may include fever, headache, myalgias, malaise and anorexia. After several days of coughing, chest wall or abdominal discomfort, which is muscular in nature, may be noted. Physical findings are generally nonspecific; examination of the chest may reveal no adventitious sounds but more commonly scattered rhonchi and occasional wheezing. Physical signs such as egophony, pleural friction rubs, or areas of dullness to percussion should suggest the presence of other diagnoses such as pneumonia or pleural effusion.

Etiology and Differential Diagnostic Features

Tracheobronchitis is most typically caused by influenza A or B virus (Table 1). Herpes simplex has been associated with necrotizing tracheobronchitis in non-immunocompromised hosts (117); this syndrome is often accompanied by refractory bronchospasm. The differential diagnosis of acute bronchitis includes nonviral infections and non-infectious etiologies such as cough-variant asthma. *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* infections cause prolonged cough. *Bordetella pertussis* infection should also be considered in the differential diagnosis of prolonged cough illness. In otherwise healthy persons, workup of acute cough should be directed toward determining the presence of pneumonia.

Treatment and Prevention

Treatment of bronchitis is primarily symptomatic with antipyretics, and cough suppression. In the absence of signs of pneumonia or documented bacterial infection such as pertussis, treatment of cough with antibacterial agents is of no benefit (118, 119).

VIRAL PNEUMONIA

Clinical Features and Syndrome Definition

The development of pneumonia is defined by the development of abnormalities of alveolar gas exchange accompanied by inflammation of the lung parenchyma, often associated with visible changes on radiologic studies. Although there can be considerable variety in the presentation of viral pneumonia depending on the age and immunologic competence of the host and the specific viral pathogen, there are certain general features of viral pneumonias. Physical findings are often nonspecific. The patient generally appears acutely ill, conjunctivitis and rhinitis may be noted, and the trachea may be somewhat tender if accompanied by viral tracheitis. Chest exam reveals increased respiratory rate, diffuse rales, and often wheezes. The sputum is relatively scant, generally shows few polymorphonuclear leukocytes, and Gram stain usually reveals minimal numbers of bacteria. The clinical presentation of viral pneumonia in children typically includes fever and lower respiratory tract signs and symptoms, such as difficulty breathing, nonproductive cough, and physical findings of wheezing or increased breath sounds. Young infants may present with apneic episodes with minimal fever. The clinical presentation may be dominated by the associated croup or bronchiolitis, which are frequently present.

A number of underlying conditions may increase the risk or severity of viral pneumonia. These features have been identified most clearly for influenza but probably impact the severity of other forms of viral pneumonia. Underlying cardiopulmonary diseases, such as valvular heart disease or chronic obstructive pulmonary disease, are well-recognized risk factors for viral pneumonia in adults and children. Neuromuscular conditions that impair clearance of respiratory secretions are also risk factors for influenza (120) and presumably other viral lower respiratory disease. Obesity has also been recognized as an important risk factor (121, 122). Individuals with compromised immune systems are susceptible to a range of pathogens that would not cause significant disease in immunologically intact individuals.

Pregnancy has long been recognized as a major risk for more severe influenza. The risks associated with pregnancy were dramatically demonstrated during the recent A(H1N1) pdm09 pandemic, where pregnant women were substantially over-represented among patients requiring hospitalization, ICU admission, and ventilatory support (123, 124). The increased risk of severe influenza extends throughout pregnancy and the immediate postpartum period. While the effects of pregnancy are most pronounced during pandemics, pregnancy has also been recognized as a risk factor for cardiopulmonary hospitalizations in the interpandemic period (125).

Bacterial superinfection is a common complication of viral lower respiratory tract infection, particularly in adults. The classic presentation is that of a typical episode of viral illness with more or less complete recovery, followed 2 to 14 days later by a recurrence of fever and development of cough and dyspnea (126). Chest X ray reveals lobar infiltrates, and the clinical course is typical of bacterial pneumonia. In addition, combined bacterial and viral pneumonia, with clinical features of each, are common. Bacterial superinfection of viral pneumonia can occur with many bacteria, but the most common bacterium responsible for bacterial pneumonia complicating influenza is *Streptococcus pneumoniae*. There are also increases in the relative frequency of *Staphylococcus aureus*, including methicillin-resistant *S. aureus* (MRSA) and *Hemophilus influenzae* (127).

The impact of viral pneumonia and the spectrum of associated viral agents are highly dependent on the age group and immune status of the host. Further details are provided in the pathogen-specific chapters. While viruses are clearly important and frequent causes of pneumonia in young children, their role is less apparent in older children. In

healthy adults, pure viral pneumonia is less common but may be associated with a variety of viruses. Elderly adults may experience more significant lower respiratory tract signs and symptoms following infection with agents that normally cause upper respiratory tract illness in younger adults. Finally, viral pneumonia is an important cause of morbidity and mortality in individuals with compromised immune systems, with a broader spectrum of viral agents than seen in immunologically intact individuals. The manifestations of viral lower respiratory tract disease in different populations are described below.

Immunocompetent Adults

Viruses are relatively less common causes of acute pneumonia in adults, but sensitive nucleic acid detection tests suggest that viruses can be detected in as many as one-third of adults with acute pneumonia (128). Influenza has been well recognized as a cause of pneumonia in adults, primarily during seasonal epidemics. In case series of community acquired pneumonia (CAP), viruses are detected in 20% to 30% of cases, frequently in combination with bacterial pathogens (129–134). RSV is generally the most commonly detected viral agent, but essentially all of the respiratory viruses have been associated with CAP (Table 2). Clinically, cases caused by RSV are not distinguishable from those associated with other viral pathogens.

Adenoviruses have been described as causes of significant outbreaks of atypical pneumonia in military recruits and less often in civilians. Illness is typically mild and clinically resembles that due to M. *pneumoniae*, but more severe disseminated infections and deaths have been reported (135). Multiple X-ray patterns are noted; there may be large pleural effusions. Prodromal symptoms of upper respiratory infection are reported by most patients, and pharyngitis is often found on presentation. Bacterial superinfection, particularly with *N. meningitidis*, may occur. Adenovirus serotypes 4 and 7 are most often implicated, but recent reports have emphasized the emergence of a relatively rare adenovirus serotype 14 responsible for severe community acquired pneumonia in adults and children (136).

Varicella is generally more severe in adults than in children, especially among smokers. Chest radiographs taken in adults with varicella will reveal infiltrates in 10% to 20%, most frequently with a nodular infiltrate in a peribronchial distribution involving both lungs; however, the majority of these individuals are asymptomatic. More severe illness is seen occasionally, and fatal varicella pneumonia has been reported in pregnancy. The severity of the pulmonary lesions in varicella generally correlates better with the diffuseness of the rash than with findings on pulmonary exam. Following recovery from varicella, the development of diffuse pulmonary calcifications has been documented.

TABLE 2 Recovery of respiratory viruses from adults and children with community acquired pneumonia

Study	Karhu 2014 (131)	Gadsby 2016 (128)	Sangil 2012 (132)	Jain 2015 (130)	Garbino 2009 (134)	Garcia-Garcia 2012 (151)	Jain 2015 (150)
Time period	3/2008–5/2012	9/2012–2/2014	11/2009–10/2010	1/2010–6/2012	NR	9/2004–7/2010	1/2010–6/2012
Location	Finland	UK	Spain	US	SWZ	Spain	US
Population	Adults, intubated	Adults, 12% in ICU	Adults	Adults, 21% ICU	Adults, mostly transplant pts	Children <14 yo	Children (70% <4 y.o.)
Number tested	49	323	131	2259	522	884	2222
Sampling	BAL, swabs, bronchial aspirate	Sputum, tracheal aspirates	Sputum, NP swabs	Swabs, urine Ag, serology	BAL specimens	Nasal aspirates	Swabs, urine Ag, serology
Results (% positive)							
Pathogenic bacteria only	43	41	34	11	24	NT	8
Mixed bacterial/viral	39	35	19	3	2	NT	7
Viruses only	10	6	17	24	15	73	66
Percent positive for:							
Influenza A/B	2	7	5	6	2	5	7
RSV	2	1	5	3	1	31	28
PIV 1-4	2	3	2	3	3	5	7
hMPV	0	1	3	4	1	5	13
Entero/Rhino	35	13	4	9	4	19	27*
Coronavirus	4	3	2	2	5	1	5
Adenovirus	8	2	0	1	0	13	11
Bocavirus	0	0	0	NT	1	13	NT

*Also detected in 17% of healthy controls.
NR, not reported; NT, not tested.

RSV frequently causes detectably altered airway reactivity in adults (137), and on occasion, lower respiratory tract involvement becomes clinically manifest as pneumonia in otherwise healthy adults (138). RSV is being increasingly recognized as a cause of significant lower respiratory tract disease in the elderly (139). It has been estimated that 2% to 4% of pneumonia deaths among the elderly in the United States may be due to RSV (140). Parainfluenza viruses have also been reported as occasional causes of pneumonia in adults and in the elderly (141). Measles can be complicated by clinically severe pneumonitis in a small percentage of healthy adults, and bacterial superinfection is common. Diffuse pneumonitis and respiratory failure have been described in association with EBV acute mononucleosis in otherwise healthy adults.

Hantaviruses are associated with hantavirus cardiopulmonary syndrome (HCPS), characterized by the onset of severe pulmonary dysfunction after a 2- to 3-day prodrome of nonspecific influenza-like symptoms, fever, myalgias, cough, gastrointestinal symptoms, and headache (142). Coryza or upper respiratory tract symptoms suggest an alternative diagnosis. Laboratory abnormalities include leukocytosis, increased hematocrit due to hemoconcentration, and thrombocytopenia with coagulopathy. However, clinical bleeding is unusual, in contrast to other systemic hantavirus syndromes (142). Moderately elevated levels of serum lactate dehydrogenase and aspartate aminotransferase are typically seen. A variety of radiographic abnormalities have been described; those that may help to distinguish HPS from adult respiratory distress syndrome (ARDS) include early, prominent interstitial edema and nonperipheral distribution of initial airspace disease (143).

Novel human coronaviruses have been associated with severe lower respiratory tract disease and acute respiratory distress syndrome (ARDS) during outbreaks, including the severe acute respiratory syndrome (SARS) coronavirus or SARS CoV in 2003 (144), and more recently the Middle East respiratory syndrome (MERS) coronavirus, or MERS-CoV (145–147). Clinical characteristics of these illnesses are similar to those of progressive respiratory distress and hypoxia (147–149).

Children

Viruses are more commonly recognized causes of pneumonia in children than in adults (128) (Table 2). In one recent series, viruses were detected in 66% of children with radiographic pneumonia, with dual bacterial and viral pathogens detected in 7% of cases (150). The frequency of virus-associated CAP begins to decrease after age 5 years. RSV has been associated with the largest proportion of viral pneumonia in young children, particularly if accompanied by bronchiolitis (87, 150, 151) (Table 1). Bronchiolitis and pneumonia represent a spectrum of lower respiratory tract involvement with RSV virus, frequently coexist, and are not clearly distinguishable. The most typical radiographic finding is diffuse interstitial pneumonitis, although lobar or segmental consolidation are evident in about one-fourth of children with RSV lower respiratory tract disease, often involving the right upper or middle lobe.

The PIVs are second only to RSV as causes of pneumonia in this age group. As described earlier, lower respiratory tract involvement is integral to the pathophysiology of croup, but pneumonia with pulmonary infiltrates is most commonly associated with PIV-3 and 4 (152). Influenza A and B viruses are both significant causes of pneumonia in children, especially during periods of epidemic prevalence (153). In infants and children, the most frequent manifestation of influenza pneumonia is an interstitial pneumonitis similar in appearance and course to those of the other predominant viral agents of pneumonia in this age group, except that a secondary bacterial pneumonia may occur more frequently than with RSV or PIV.

Rhinoviruses have also been associated with a significant proportion of CAP in children, despite their apparent temperature sensitivity. Recent studies using sensitive PCR-based diagnostics have suggested that RV may be the second or third most common virus detected in acute pneumonia in children (150, 151, 154). However, RV is detected almost as frequently in age- and site-matched asymptomatic controls (150). Adenoviruses are also frequently isolated from children with respiratory disease and are implicated in about 10% of childhood pneumonias. However, the true impact of adenoviruses as causes of pneumonia in this age group is difficult to assess because of the long and intermittent asymptomatic respiratory shedding of these viruses in children. Hilar adenopathy on chest X ray is somewhat more common with this form of pneumonia than other types (155). Pneumonia is the most frequent serious complication of measles. Other viruses that may occasionally cause viral pneumonia in children include enteroviruses, rubella virus, and herpes simplex virus. Premature infants are at risk for pneumonia due to cytomegalovirus because of lack of maternal antibodies.

Pneumonia is the most frequent serious complication of measles. The prodrome of typical measles lasts 2 to 8 days and is characterized by fever, malaise, anorexia, cough, coryza, and conjunctivitis. Koplik's spots, which are erythematous macular lesions with central white-yellow or gray puncta, appear on the buccal or labial mucous membranes toward the end of the prodromal period. The maculopapular, erythematous eruption begins about the face and neck and progresses to involve the upper body, trunk, and extremities. The rash typically disappears after 5 to 6 days in the order in which it appeared. Defervescence and symptom improvement occur several days after the appearance of the rash, although persistent cough is common. Leukopenia is common during the prodromal and early exanthematous stages of measles. Pronounced leukopenia (less than 2,000 cells/mm^3) is associated with a poor prognosis. The development of neutrophilic leukocytosis suggests the possibility of bacterial superinfection or other complications.

Immunocompromised Individuals

Individuals with diminished host immunity may develop severe, life-threatening pulmonary infections with the entire spectrum of RNA and DNA viruses, including both viruses that are typical causes of lower respiratory tract disease in normal hosts and other more opportunistic viral pathogens (Table 1). DNA viruses have received the most recognition in this regard.

CMV is a frequent cause of severe pneumonitis in immunosuppressed individuals, particularly transplant recipients (156). The highest risk in the transplant population is 1 to 3 months post-transplantation, with the peak incidence at 8 weeks' post-transplantation. Diffuse interstitial pneumonitis is the most frequent manifestation, but multiple other radiographic presentations have been reported, including nodular infiltrates. Multiple associated findings are present in severe infection and reflect the disseminated nature of the infection; the presence of neutropenia, abnormalities of liver function tests, and mucosal ulcerations may be clinical clues to the diagnosis.

Herpes simplex virus pneumonia has been reported largely in immunocompromised or debilitated individuals. These cases are variably preceded by clinically evident mucocutaneous disease. The majority of cases present as a focal pneumonia as a result of contiguous spread from the upper respiratory tract; diffuse interstitial disease resulting from hematogenous spread occurs in up to 40% of cases (157). Risk groups include neonates, transplant recipients, burn patients particularly with inhalation injury, and those who have experienced prolonged mechanical ventilation, cardiothoracic surgery, or trauma.

Varicella-zoster virus is an important problem in individuals with hematological malignancies and others with iatrogenic immunosuppression, with the greatest risk seen in organ transplantation. Prolonged fever and recurrent crops of lesions are predictors of visceral dissemination, and pneumonia is generally seen in this setting. Pulmonary manifestations may include pleuritic chest pain due to vesicular lesions of the pleura, and, as also true in normal hosts, the chest radiographs may demonstrate diffuse nodular lesions.

Adenoviruses are significant causes of morbidity and mortality in immunocompromised patients, particularly after transplantation. In contrast to infection in normal hosts, infection in immunocompromised subjects tends to be disseminated, with isolation of virus from multiple body sites including lung, liver, gastrointestinal tract, and urine (158). In addition, the spectrum of serotypes includes both those found in immunocompetent individuals as well a markedly increased frequency of isolation of higher-numbered serotypes found rarely in immunologically normal subjects (159).

Common respiratory viruses have also received increasing recognition as potential causes of significant morbidity and mortality in this population (160). RSV has been well recognized as a cause of severe pneumonia in recipients of bone marrow (161) and solid organ transplantation (162). Nosocomial transmission of RSV in this setting has been well documented and may be the source of many infections in this susceptible population. The illness typically begins with nondescript upper respiratory symptoms that progress over several days to severe, life-threatening lower respiratory tract involvement. Mortality of 50% or higher is typical if pneumonia supervenes, particularly if disease occurs in the pre-engraftment period (163). Parainfluenza viruses have also been reported as an infrequent lower respiratory tract pathogen in both solid-organ and bone marrow transplantation. PIV-3 has been most common serotype isolated, but all four serotypes have been implicated (160). Influenza virus may also cause severe disease in transplant recipients (164) and patients with leukemia. Rhinoviruses and coronavirus infections in this population are also common but tend to be associated less frequently with lower respiratory tract disease (165). In transplant recipients, infections with community respiratory viruses may result in long-term impairment of respiratory function (166).

Measles giant cell pneumonia is a severe, usually fatal form of pneumonia in immunosuppressed individuals, including those who are severely malnourished. Most cases have occurred in those with hematological or other malignancies or in individuals with AIDS (167). Such hosts do not mount the cellular immune responses involved in the pathogenesis of measles rash or other typical manifestations of measles, and a high index of suspicion must be maintained (167). Giant cell pneumonia also occurs in significantly malnourished individuals. Multinuclear giant cells with intranuclear inclusions are seen and may be demonstrable in fluid obtained by bronchoalveolar lavage.

Diagnosis

Evaluation of the specific cause of acute pneumonia, and in particular, attribution of pneumonia to a particular viral etiology, is complicated by difficulty in obtaining appropriate samples of lower respiratory tract secretions, and the frequent asymptomatic shedding of some viruses, such as rhinovirus, herpes viruses, or adenoviruses in the upper respiratory tract.

The clinical presentation, epidemiology, and presence of associated features such as rash, may provide strong clues regarding the specific viral etiology of pneumonia, especially in children. However, distinguishing purely viral from bacterial or combined viral and bacterial lower respiratory tract disease remains an extremely difficulty challenge. This is a particularly important goal in reducing the unnecessary use of antibacterial therapy, and reducing rates of antibiotic resistance and complications such as *Clostridium difficile*.

Highly sensitive multiplex nucleic acid detection tests are now widely available in well-resourced settings and increasingly used to detect respiratory viruses in both upper and lower respiratory tract samples (see Chapter 15). Interpreting the results of such tests is complicated by the reality that detection of a virus does not rule out the presence of a coexisting bacterial infection nor represent compelling evidence that antibacterial therapy is not needed.

Radiologic findings also do not reliably distinguish viral from bacterial, or between viral causes of pneumonia (168). Recently, a number of biomarkers have been proposed for this purpose. The most widely used is probably the serum procalcitonin test, with the presence of a high pro-calcitonin associated with a higher likelihood of bacterial infection (169). The C reactive protein (CRP) is also sometimes used in the same way (170). However, there is debate whether the sensitivity and specificity of these tests is in the range to be able to guide decision-making for antimicrobial use (171). Recently, the use of a combination of markers, essentially developing a transcriptional profile of responding cells, has been demonstrated to have better sensitivity and specificity in this regard (172), and may pave the way for more accurate determination of the cause of pneumonia.

Pathogenesis

The pathogenesis of viral infections of the lower respiratory tract can be conveniently considered in terms of infections initiated in and primarily confined to the respiratory tract, such as with influenza or RSV; processes in which infection is initiated in the respiratory tract with subsequent systemic manifestations, such as in measles or varicella; and processes where respiratory tract involvement is secondary to a systemic infection, such as with cytomegalovirus. Each of these situations may lead to what is recognized clinically as a viral pneumonia. The general features of primary viral pneumonia are discussed below using influenza as a model, and pathogenesis of other forms of viral pneumonia is discussed briefly in comparison.

In primary viral pneumonia, virus infection reaches the lung either by contiguous spread from the upper respiratory tract or by inhalation of small particle aerosols. Infection initially occurs in ciliated respiratory mucosal epithelial cells of the trachea, bronchi, and lower respiratory tract and leads to widespread destruction of these cells. The mucosa is

hyperemic, and the trachea and bronchi contain bloody fluid. Tracheitis, bronchitis, and bronchiolitis are seen, with loss of normal ciliated epithelial cells. Submucosal hyperemia, focal hemorrhage, edema, and cellular infiltrate are present. The alveolar spaces contain varying numbers of neutrophils and mononuclear cells admixed with fibrin and edema fluid. The alveolar capillaries may be markedly hyperemic with intra-alveolar hemorrhage. Acellular, hyaline membranes line many of the alveolar ducts and alveoli (see Figure 10 in Chapter 43). Pathologic findings seen by lung biopsy in nonfatal cases during non-pandemic situations are similar to those described in fatal cases (173).

The pathologic changes in the lower respiratory tract in children with viral pneumonia due to RSV and PIV are nonspecific and include epithelial necrosis with bronchiolar mucus plugging and widespread inflammation and necrosis of lung parenchyma, and severe lesions of the bronchial and bronchiolar mucosa as well (99) (see Figures 4 and 5 in Chapter 37). In fatal cases of RSV pneumonia in children, hemorrhagic pneumonia with peribronchial mononuclear infiltration and cytoplasmic inclusion bodies in epithelial cells are seen. Giant cell pneumonia with virally induced multi-nucleated syncytial cells may be seen in RSV, PIV, or measles infections in immunocompromised hosts.

Bacterial superinfection is a well-recognized complication of viral pneumonia and accounts for a large proportion of the morbidity and mortality of viral lower respiratory tract disease, especially in adults. Consequently, the spectrum of disease and pathophysiology of bacterial superinfection has been studied intensively, and a number of factors in viral respiratory disease have been identified which could play a role in increasing the risk of bacterial infection. The disruption of the normal epithelial cell barrier to infection and loss of mucociliary clearance undoubtedly contribute the enhancement of bacterial pathogenesis (174). In addition, increased adherence of bacteria to virus-infected epithelial cells has been demonstrated. Polymorphonuclear leukocytes and mononuclear cells are susceptible to abortive infection by some respiratory viruses with resulting decreased function which may also contribute to enhanced bacterial infection (175). Virus-induced impairment of repair functions has also been proposed (176).

Infection with influenza, RSV, PIV, and adenoviruses is usually limited to the respiratory tract by mechanisms which are not completely clear. In contrast, respiratory tract infection with measles or varicella virus leads to dissemination and systemic manifestations. In more severe cases of varicella, vesicles may be found within the tracheobronchial tree and on pleural surfaces. Microscopic examination demonstrates interstitial pneumonitis with edema, and intranuclear inclusion bodies within septal cells, and peribronchiolar inflammation.

The Hantavirus pulmonary syndrome represents an additional example of a viral infection which involves the lung as part of a systemic infection. The pathogenesis of HPS involves extensive infection of endothelial cells throughout the body, which is particularly intensive within the vascular endothelial cells of the lung (177). Abundant viral antigen and nucleic acid can be detected within these cells. Microscopic examination of the lung reveals mild to moderate interstitial pneumonitis with variable degrees of congestion, edema, and mononuclear cell infiltration (see Figure 4 in Chapter 44). The cellular infiltrate is composed of a mixture of small and large mononuclear cells, which consist predominantly of T-lymphocytes, and macrophage/monocytes. The picture is one of immune mediated capillary leak and not of cell necrosis or inflammatory pneumonitis. High levels of cytokines have been detected in the blood and likely mediate the endothelial damage.

There are several features of CMV pneumonitis in the transplant setting that suggest that both host and viral factors interact in pathogenesis (178). CMV pathogenicity is enhanced in transplant recipients and frequently occurs at the site of the transplanted organ. The risk of CMV pneumonitis is also highest in individuals at the highest risk for graft versus host disease (179).

Treatment and Prevention

Therapy of viral pneumonia is dependent on the severity of disease, the age and immune status of the host, and the specific causative viral agent. General supportive measures, particularly the management of hypoxia, are critically important, and some patients have required high frequency ventilation or extracorporeal membrane oxygenation. Although inflammatory responses contribute to the pathogenesis of viral pneumonia, early corticosteroid treatment is generally associated with worse outcomes (180–182) Since mixed viral-bacterial infections or bacterial superinfections are common, antibacterial agents may be required as indicated by appropriate microbiologic studies.

Antiviral therapy should be guided by the results of diagnostic tests (Table 3). The neuraminidase inhibitors zanamivir and oseltamivir are active against both influenza A and B viruses (183). It should be noted that these agents have mostly been studied in uncomplicated influenza in healthy adults, where the main effect is in reduction of the duration of illness. However, observational studies in hospitalized patients (184, 185) have shown the mortality benefit of early oseltamivir therapy, and surveillance data suggesting that therapy as late as 5 days improved survival of hospitalized patients (186). Inhaled zanamivir may be difficult to reliably and safely deliver in severe influenza, but an intravenous formulation has been used with apparent benefit, including in infections due to oseltamivir resistant A (H1N1) viruses. The neuraminidase inhibitor peramivir has also recently been approved for intravenous use in the United States, although a small study did not demonstrate benefit in hospitalized patients (187). Although the M2 inhibitors are highly effective drugs for the prophylaxis and therapy of influenza A virus, currently circulating seasonal influenza A viruses are uniformly resistant to these agents (188). However, there may be a role for these drugs in combination therapy of influenza (189), and studies to evaluate this in humans are in progress.

The only option currently available for the other RNA viruses is ribavirin, but there is little evidence of efficacy of this agent for treating established viral pneumonia (see bronchiolitis, above). In immunocompromised hosts, treatment of RSV pulmonary infection associated with respiratory failure has not been successful. One approach that appears promising is treatment with ribavirin, possibly in combination with immunoglobulin, early in the illness when URI symptoms predominate (160). Controlled trials in parainfluenza virus infection are not available, although anecdotal reports suggest potential efficacy (190). Limited controlled trials have suggested that aerosolized ribavirin may reduce the severity of symptoms in children with measles, and some immunocompromised patients with measles pneumonia have done well following treatment with aerosolized (167) or intravenous forms of the drug (191). Intravenous ribavirin is effective in the treatment of hemorrhagic

TABLE 3 Therapies of potential benefit in viral pneumonia

Viral Etiology	Potential Therapies	Comments
Respiratory syncytial virus	Ribavirin	May be considered for use in high-risk or severely ill children. Premptive use in transplant patients.
	Palivizumab (Synagis)	Effective prevention of RSV bronchiolitis/pneumonia in high-risk premature infants who lack maternal antibody.
Parainfluenza virus	DAS-181	Case reports of efficacy in hematologic transplant, investigational, may be available for compassionate use.
	Ribavirin	Case reports of efficacy of IV or oral ribavirin in parainfluenza virus infection, aerosolized ribavirin not recommended. Addition of IVIG may be helpful.
Influenza virus	Neuraminidase inhibitors Oseltamivir (oral) Zanamivir (intravenous) Peramivir (intravenous)	Timely oseltamivir therapy associated with reduced rates of pneumonia development and mortality in hospitalized patients. Indicated for severe or progressive disease and in high-risk patients. IV zanamivir is active against most oseltamivir-resistant variants. IV peramivir also available.
Measles virus	Ribavirin	Aerosolized or IV ribavirin may shorten the duration of illness in children with measles. Use in measles pneumonia is unproved.
	IVIG	IVIG may decrease risk of measles when administered to susceptible individuals, and may decrease symptoms in those infected.
Adenovirus	Cidofovir, brincidofovir	Cidofovir is active *in vitro* and multiple case reports suggest efficacy. Brincidofovir has less renal toxicity; recent clinical trial suggests efficacy against adenovirus pneumonia in transplant patients.
Herpes simplex virus	Acyclovir (Valacyclovir, Famciclovir)	Controlled trials have demonstrated efficacy of acyclovir in a variety of HSV diseases. Cross resistance between agents.
	Foscarnet, cidofovir	May be useful for treatment of herpes viruses resistant to acyclovir.
Varicella-zoster virus	Acyclovir (Valacyclovir, Famciclovir)	Demonstrated efficacy of IV acyclovir in varicella and in varicella pneumonia, must use relatively high doses.
	Foscarnet	May be useful for management of acyclovir-resistant cases.
Cytomegalovirus	Ganciclovir	Clinical efficacy in CMV pneumonitis in AIDS and solid-organ transplantation. In bone marrow transplant patients, efficacious when combined with IVIG.
	Foscarnet, cidofovir	Predominant use in gancyclovir-resistance or in individuals who cannot tolerate gancyclovir due to hematological toxicity.

Note: Listing of potential therapies only, not to be considered a recommendation for use. Please see pathogen-specific chapters and Chapter 14 on antivirals for respiratory viruses for more detailed treatment recommendations.

fever with renal syndrome, but does not appear to be useful for treatment of the hantavirus pulmonary syndrome (192).

An experimental agent that has shown some promise in treatment of severe parainfluenza virus infection in immunosuppressed hematopoietic stem cell transplant recipients is the sialidase construct DAS-181 (193). The drug is administered by inhalation, and mechanism of action is thought to be removal of sialic acid receptors from the host respiratory tract. Two investigational RSV antivirals, the fusion inhibitor presatovir (GS-5806) and the polymerase ALS-8176, have shown promising activity in experimentally induced RSV infections in adults (194, 195) and are undergoing clinical trials in serious RSV infections at present.

Acyclovir is active *in vitro* against herpes simplex virus types 1 and 2 and against varicella-zoster virus, but it does not have clinically useful activity for treatment of cytomegalovirus or Epstein-Barr virus disease. Although controlled clinical trials of this drug in herpes simplex pneumonia have not been conducted, the drug has proven clinical efficacy in other herpesvirus infections and would be indicated in any serious HSV lower respiratory tract infection. Acyclovir is also effective in the therapy of varicella, and intravenous acyclovir has been effective when initiated early in the course of varicella pneumonia (196). The related drugs valacyclovir, famciclovir, and penciclovir are similar to acyclovir in their spectrum of activity against herpes and varicella viruses. Viruses resistant to the activity of these drugs have been isolated from treated immunocompromised patients, and may be susceptible to the antiherpes drug phosphonoformic acid (foscarnet).

Guidelines for management of CMV disease in transplant patients have recently been published (197, 198). Transplant candidates should be screened for evidence of CMV immunity, and CMV-seronegative recipients of transplants from CMV-positive donors are at the highest risk of CMV disease. One strategy for prevention of CMV disease is to provide prophylaxis with ganciclovir or valgancyclovir during the period of highest risk, over the first 3 to 6 months after transplantation. Alternatively, some centers favor a preemptive therapy approach, where patients are monitored with serial PCR and antiviral therapy is initiated when CMV PCR becomes positive and reaches a predefined threshold.

Once CMV pneumonitis is established, particularly in allogeneic bone marrow transplant patients, it can be very difficult to treat. Ganciclovir is highly active against CMV *in vitro*, and intravenous ganciclovir therapy is generally recommended in cases of severe disease, although the orally available drug valgancyclovir can be used in less-severe cases. Cidofovir and foscarnet are considerations for CMV resistant to ganciclovir. The combination of ganciclovir therapy and intravenous CMV immune globulin or IVIG can reduce mortality in stem cell transplant recipients (199, 200) and is generally recommended in this situation.

Antiviral treatment of proven value for adenovirus infection is not available. Cidofovir is active against adenovirus *in vitro*, and there are several case reports or case series of successful therapy of adenovirus infection in immunocompromised patients with cidofovir (201, 202). However, cidofovir has substantial renal toxicity, which limits its utility in this application. A newly derived series of lipid ester derivatives of cidofovir are orally bioavailable and have less renal toxicity. One of these agents, brincidofovir, has shown preliminary evidence of efficacy against adenovirus infections in bone marrow transplant recipients (203–205).

Although recent years have witnessed a significant increase in the spectrum and potency of available antiviral agents, drug therapy of viral pneumonia remains burdened by the toxicity of drugs, the development of antiviral resistance, and the complex pathogenesis of many viral syndromes in which viral replication is only part of the disease process. Vaccines of variable effectiveness currently exist for influenza, measles, and varicella virus. Development of additional effective vaccines for the viral pathogens causing pneumonia will contribute to the control of this important problem.

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Viral Infections of the Central Nervous System

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3

Central nervous system (CNS) symptoms (headache, lethargy, impaired psychomotor performance) are frequent components of viral infections; however, viral infections of the CNS occur infrequently and most often result in relatively benign, self-limited disease. Nevertheless, these infections have tremendous importance because of their potential for causing neurologic damage and death. The CNS is exquisitely sensitive to metabolic derangements and tissue injury. Clinical recovery is slow and often incomplete (1, 2). Patient history, while frequently suggestive of a diagnosis, remains an unreliable method for determining the specific etiology of CNS disease (1, 3). Tumors, infections, and autoimmune processes in the CNS often produce similar signs and symptoms (3). Different diseases may share common pathogenic mechanisms and therefore result in a similar clinical presentation. Furthermore, understanding disease pathogenesis provides a rational basis for the development of therapeutics including antivirals and strategies for the prevention of viral CNS infections.

The definitions of viral CNS disease are often based on both virus tropism and disease duration. Inflammation may occur at multiple sites within the CNS and accounts for the myriad clinical descriptors of viral neurological disease. Inflammation of the spinal cord, leptomeninges, dorsal nerve roots, or nerves results in *myelitis*, *meningitis*, *radiculitis*, and flaccid paralysis and *neuritis*, respectively. *Aseptic meningitis* is a misnomer frequently used to refer to a benign, self-limited viral infection causing inflammation of the leptomeninges (4). The term hinders epidemiologic studies because the definition fails to differentiate between infectious (fungal, tuberculous, viral, or other infectious etiologies) and noninfectious causes of meningitis. *Encephalitis* refers to inflammation of parenchymal brain tissue and is frequently associated with *encephalopath*y or depressed level of consciousness, altered cognition, and frequently focal neurological signs. *Acute encephalitis* occurs over a relatively short period of time (days) while *chronic encephalitis* presents over weeks to months. The temporal course of *slow infections* of the CNS (kuru, visna, variant Creutzfeldt-Jacob disease) overlaps with the chronic encephalitides. Slow infections of the CNS are distinguished by their long incubation period combined with a slow replication rate, eventually resulting in death or extreme neurological disability over months to years (5).

Viral disease in the CNS can also be classified by pathogenesis. Neurological disease is frequently categorized as either *primary* or *postinfectious*. *Primary encephalitis* results from direct viral entry into the CNS that produces clinically evident cortical or brainstem dysfunction (4). Subsequent damage results from a combination of viral-induced cytopathic effects and host immunopathologic responses. Viral invasion, however, remains the initiating event (4). The parenchyma exhibits neuronophagia and the presence of viral antigens or nucleic acids A *postinfectious* or *parainfectious encephalitis* produces CNS signs and symptoms, either following or temporally associated with a systemic viral infection, respectively, without evidence of direct viral invasion in the CNS. Pathology specimens show demyelination and perivascular aggregation of immune cells, without evidence of virus or viral antigen, leading some to hypothesize an autoimmune etiology (4).

Meningitis and encephalitis represent separate clinical entities; however, a continuum exists between these distinct forms of disease. A change in a patient's clinical condition can reflect disease progression through involvement of different regions of the brain. Therefore, in many cases it is difficult to accurately and prospectively predict the etiology and eventual extent of CNS infection. Epidemiologic data (patient demographics and immune status; season, geographical location; vector, animal, and other exposures) in many cases provide clues to the etiology of the illness. An overview is difficult, as each pathogen fills a different ecological niche with unique seasonal, host, and vector properties (Tables 1 and 2 a, b) (4). Instead it is useful to analyze the prototypes of viral CNS infection, meningitis, and encephalitis and the approach to patients with presumed viral infections of the CNS.

VIRAL MENINGITIS

Epidemiology

Acute viral meningitis and meningoencephalitis represent the majority of viral CNS infections and frequently occur in epidemics or in seasonal patterns (4, 6). While there have been changes in the epidemiology of viral meningitis in North America because of the recent introduction of West Nile virus, enteroviruses cause the majority of viral

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TABLE 1 DNA viruses: type of disease, epidemiologic data, and pathogenesis of viral infections of the CNS

Viral agent	CNS disease	Temporal course	Transmission	Pathway to CNS	Relative frequency	Laboratory confirmation
Herpetoviridae						
Herpes simplex virus HSV type 1	Encephalitis	Acute (congenital) Sporadic (latent)	Human	Neuronal, Blood Neuronal	+++	PCR—CSF Cell culture—brain biopsy sample
HSV type 2	Meningitis Encephalitis	Primary, recurrent Acute (congenital)	Human	Neuronal, Blood Blood, Neuronal	++	Cell culture—genital, rectal, skin PCR—CSF
Cytomegalovirus (CMV)	Encephalitis (neonate and immunosuppressed)	Acute Subacute	Human	Blood	++	PCR Cell culture—brain biopsy or CSF
Epstein-Barr virus (EBV)	Encephalitis Meningitis Myelitis, Guillain-Barré syndrome	Acute	Human	Blood	+	PCR
Varicella zoster virus (VZV)	Cerebellitis, Encephalitis, Meningitis, Myelitis	Postinfectious (acute) Latent reactivation (zoster)	Human	Blood Neuronal	++	PCR, clinical findings, cell culture from a lesion, brain biopsy or, rarely, necroscopy
Human herpesvirus 6 (HHV-6)	Encephalitis, febrile seizures, latent form?	Acute	Human	Blood		PCR
B virus (Cercopithecine herpesvirus 1)	Encephalitis	Acute	Animal bite and human	Blood	+	Culture, PCR (high frequency of detection, unknown significance)
Adenoviridae						
Adenovirus	Meningitis, encephalitis	Acute	Human	Blood	+	Cell culture of CSF or brain
Poxviridae						
Vaccinia	Encephalomyelitis	Postinfectious	Vaccine	Blood	Presumed extinct	Recent vaccination

Frequency: +++ = Frequent, ++ = Infrequent, + = Rare, ? = Unknown.

meningitis infections. Arboviruses constitute the second most common cause of viral meningitis in the United States (1, 7–9). Mumps virus remains an important cause of viral CNS disease in countries that do not immunize against this virus. The risk of meningitis from natural mumps infection outweighs the risk of aseptic meningitis associated with the vaccine; nonetheless, not all countries vaccinate against mumps (1, 10). There are more than 74,000 cases of viral meningitis a year in the United States (11). Most cases occur from the late spring to autumn months reflecting increased enteroviral and arboviral infections during these seasons (1, 11). A retrospective survey performed in the 1980s found that the annual incidence of "aseptic meningitis" was approximately 10.9/100,000 persons or at least four times the incidence passively reported to the CDC during the period (6).Virus was identified in only 11% of patients in this study. With the advent of improved nucleic acid-based diagnostic methods, etiologic diagnosis rates now approach 50% to 86% (1, 12, 13).

The pathogenesis of viral meningitis is incompletely understood. Inferences regarding the pathogenesis of viral meningitis are largely derived from data on encephalitis, experimental animal models of meningitis, and clinical observations (4). Viruses use two basic pathways to gain access to the CNS: hematogenous and neuronal spread. Most cases of viral meningitis likely occur following a high-titer secondary viremia although some, such as meningitis associated with genital herpes or herpes zoster, are related to neuronal routes. A combination of host and viral factors combined with seasonal, geographic, and epidemiologic probabilities influence the proclivity to develop viral CNS infection. For example, arboviral infections occur more frequently in epidemics and show a seasonal variation, reflecting the prevalence of the transmitting vector (1, 14). Enteroviral meningitis occurs with greater frequency during the summer and early autumn months in temperate climates, reflecting the seasonal increase in overall enteroviral infections. Enteroviral infections also exemplify the difference host physiology plays in determining the extent of viral disease. In children less than 2 weeks of age, enterovirus infections can produce a severe systemic infection, including meningitis or meningoencephalitis (11). Ten percent of neonates with systemic enteroviral infections die, while as many as 76% are left with permanent sequelae. In children over 2

TABLE 2a RNA viruses: type of disease, epidemiologic data, and pathogenesis of viral infections of the CNS

Viral taxonomy	CNS disease	Case fatalities	Vector	Geographic distribution
Togaviridae—Alphavirus (Arbovirus)				
Western equine encephalitis virus	Meningitis, encephalitis	3%–10%	Mosquitoes, birds	United States—west of Mississippi river
Eastern equine encephalitis virus		>30%		United States—Atlantic and Gulf Coast states
Venezuelan equine encephalitis virus		<1%	Mosquitoes, horses	Central and South America, Southwestern United States and Florida
Flaviviridae—Flavivirus (Arbovirus)				
Japanese encephalitis virus	Meningitis, encephalitis	25%	Mosquitoes, swine, birds	Japan, China, Korea, Taiwan, S.E. Asia, India, Nepal
St. Louis encephalitis virus		7%		United States
West Nile fever virus		Higher attack rate in elderly 11%–33%		Africa, Middle East, India, Eastern Europe, recently imported into United States, spreading rapidly
Murray Valley virus	Encephalitis	20%–60%		Australia
Tick-borne encephalitis virus (TBE complex)		20%	Ticks, unpasteurized milk	Eastern Russia and Central Europe
Bunyaviridae—(Arbovirus)				
California (La Cross) encephalitis virus	Meningitis, encephalitis	<1%	Mosquitoes, rodents	Northern Midwest and Northeastern U.S.
Reoviridae—Coltivirus (Arbovirus)				
Colorado tick fever virus	Meningitis, encephalitis	<1%	Ticks, rodents	U.S. Rocky Mountains, Pacific Coast states Coast States United States
Picornaviridae—(Enterovirus)				
Poliovirus	Meningitis, myelitis	4.5%–50%[a]	Fecal oral	Worldwide
Coxsackievirus Echovirus	Meningitis, meningoencephalitis, myelitis	Rarely[b]		
Paramyxoviridae—**(Exanthematous virus)**				
Measles virus	Encephalitis, SSPE	15%	Postinfectious Blood	Worldwide
Mumps virus	Meningitis, encephalitis, myelitis	<1%	Blood	
Orthomyxoviridae—**(Upper respiratory virus)**				
Influenza viruses	Encephalitis	<1%	Postinfectious	Worldwide
Rhabdoviridae				
Rabies	Encephalitis, encephalomyelitis	~100%	Mammal	Worldwide
Retroviridae				
Human immunodeficiency virus type 1 (HIV 1)	Encephalopathy, encephalitis, leukoencephalopathy	Majority	Human	Worldwide
Arenaviridae				
Lymphocytic choriomeningitis virus	Meningitis, encephalitis	<2.5%	Rodent	Worldwide

[a]Fatality from poliomyelitis is increased in sporadic cases. With vaccination, the epidemic forms of polio have decreased as has morbidity. In turn, the calculated case fatality rate in the United States has increased as sporadic and vaccine-associated disease has increased relative to the number of cases of disease.
[b]Rarely fatal except in nenonates and agammaglobulinemic patients where fatality rates can approach 50% even with treatment.

TABLE 2b RNA viruses: type of disease, epidemiologic data, and pathogenesis of viral infections of the CNS

Viral taxonomy	Disease pattern	Pathway to CNS	FREQ	Laboratory confirmation
Togaviridae—Alphavirus (Arbovirus)				
Western equine encephalitis virus	Epidemic	Blood	++	Serologic titers (HI, CF, N, IFA), viral antigen detection in brain, culture (rare)
Eastern equine encephalitis virus	Sporadic		+	Viral culture or antigen detection in brain, serologic titers (HI, CF, N, IFA), CSF IgM ELISA
Venezuelan equine encephalitis virus	Sporadic, epidemic		+	Serologic titers (HI, CF, N, IFA), CSF IgM ELISA
Flaviviridae—Flavivirus (Arbovirus)				
Japanese encephalitis virus	Epidemic, endemic	Blood	+++	Peripheral blood ELISA, serologic titers (HI, CF, N, IFA), CSF antigen test/PCR
St. Louis encephalitis virus			+++	CSF IgM ELISA, serologic titers (HI, CF, N, IFA), PCR, culture (rare)
West Nile fever virus			+++	Culture (rare), serology (HI, IFA), PCR
Murray Valley virus			++	Viral culture, serologic titer (HI, CF, N)
Tick-borne encephalitis virus (TBE complex)	Epidemic, sporadic		++	Serologic titer (HI, CF, N), IgM ELISA PCR
Bunyaviridae—(Arbovirus)				
California (La Crosse) encephalitis virus	Endemic	Blood	+++ (LCV) + (CEV)	Viral culture, CSF IgM ELISA, serologic titers (HI, CF, N, IFA), CIE, PCR
Reoviridae—Coltivirus (Arbovirus)				
Colorado tick fever virus	Endemic	Blood	+	Antigen detection on RBC membrane, viral culture, serologic titers (HI, CF, N, IFA)
Picornaviridae—(Enterovirus)				
Poliovirus	Endemic	Blood and neuronal	++	Viral culture CSF or brain, viral culture from other site, serologic testing for some serotypes, PCR
Coxsackievirus		Blood	+++	
Echovirus			+++	
Paramyxoviridae—(Exanthematous virus)				
Measles virus	Sporadic	Blood	++	Serology, ELISA, clinically
Mumps virus			+++	CSF viral culture
Orthomyxoviridae—(Upper respiratory virus)				
Influenza viruses	Sporadic	Blood	+	Viral culture from another site
Rhabdoviridae				
Rabies	Sporadic	Neuronal	+++	Antigen detection in brain serologic tests, (IFA, CF, HA, CIE), viral culture.
Retroviridae				
Human Immunodeficiency virus type 1 (HIV 1)	?Progressive	Blood	++	PCR CSF/autopsy samples/MRI findings, isolation, *in situ*, antigen detection
Arenaviridae				
Lymphocytic choriomenigitis virus	Sporadic	Blood	+	CSF, blood culture, urine culture, serology congenital infection

HI, hemagglutination inhibition; CF, complement fixation; NA, neutralizing antibody titer; CIE, counterimmunoelectrophoresis; IFA, immunofluorescent antibody; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; MRI, magnetic resonance imaging.
Frequency: +++ = Frequent; ++ = Infrequent; + = Rare; ? = Unknown.

weeks of age, however, enteroviral infections are rarely associated with severe disease or significant morbidity with the exception of enterovirus D68, for which substantial morbidity has been documented (11).

The sequence of viral hematogenous spread to the CNS is illustrated in Figure 1 (4). A virus must first bypass or attach to and enter host epithelial cells to produce infection. Virus then spreads and initially replicates in the regional lymph nodes (e.g., measles, influenza) or alternatively enters the circulatory system where it seeds other tissues (e.g., arboviruses, enteroviruses, varicella) (4). Primary viremia allows virus to seed in distant locations of the body and frequently marks the onset of clinical illness. In rare circumstances such as disseminated neonatal herpes simplex

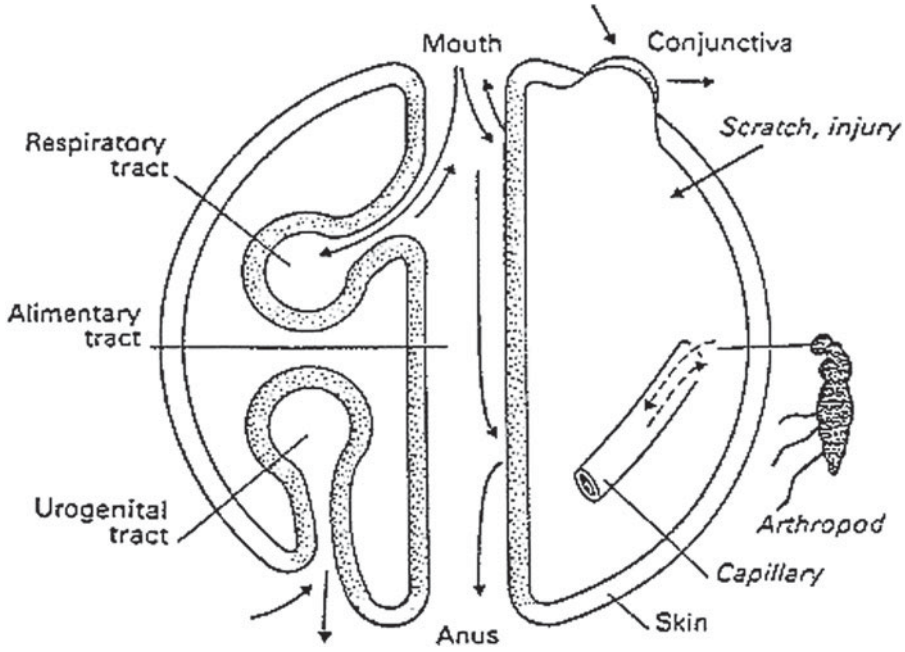

FIGURE 1 Body surfaces as sites of virus infection and shedding.

virus (HSV) infection, viruses infect the CNS during primary viremia (15, 16); however, most viral infections involve an intermediate organ prior to reaching the CNS. The liver and spleen provide ideal locations for secondary viral replication and hematogenous spread because of their highly vascular nature. Secondary viremia may result in high titers of virus in the bloodstream for prolonged periods of time, facilitating viral CNS spread. The pathophysiology of viral transport from blood to brain and viral endothelial cell tropism are poorly understood. Virus infects endothelial cells, leaks across damaged endothelia, passively channels through endothelium (pinocytosis or colloidal transport), or bridges the endothelium within migrating leukocytes (4). This trans-endothelial passage occurs in vessels of the choroid plexus, meninges, or cerebrum, as depicted in Figure 2.

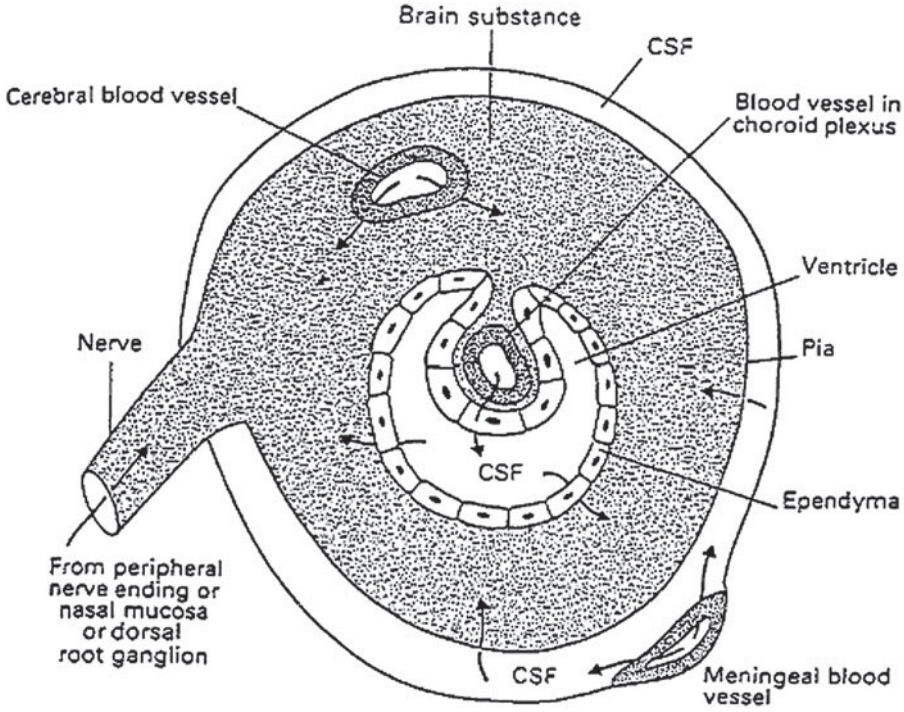

FIGURE 2 Routes of viral invasion of the central nervous system. CSF, cerebrospinal fluid.

Numerous barriers and host defenses limit viral dissemination to the CNS. The skin and mucosal surfaces possess mechanical, chemical, and cellular defenses that protect the cells from viral infection (4). Leukocytes and secretory factors (interleukins, interferons, antibodies) further augment these defenses and help eliminate viruses that bridge the epithelial layer. Local innate immune responses are crucial in limiting systemic viral infection; a swift inflammatory response can limit viremia. In the liver and spleen, the high degree of parenchymal contact and large number of fixed mononuclear macrophages provide an excellent opportunity for host eradication of viremia (4). The blood-brain or blood-CSF barrier, a network of tight endothelial junctions sheathed by glial cells that regulate molecular access to the central nervous system, further limits viral access to the CNS (17, 18).

Viral meningitis is a relatively benign, self-limited illness and pathological specimens are rarely available for study (11). The CSF, however, is frequently sampled and demonstrates a mononuclear immune cell response to most viral infection. Certain viral infections, most notably mumps and some enterovirus infections, elicit a CSF polymorphonuclear cell response early during disease. The initial CSF cellularity mimics bacterial meningitis and later shifts to mononuclear cell predominance. Viral antigen presentation by mononuclear histiocytes stimulates the influx of immune cells. Recruited immune cells release soluble factors (interleukins, vasoactive amines) that mobilize other cells and change the permeability of the blood-brain barrier (1, 19). The viral etiology and type of CNS disease (meningitis vs. encephalitis) can produce differences in CSF IFN γ, IL2, IL6, IL12, procalcitonin, and lactate levels (20–24). While research data suggests that these may be used to differentiate CNS disease, CSF cytokine measurements are not a routine commercial diagnostic method and are often limited to research institutions. Furthermore, these biomarkers are also elevated in autoimmune CNS disease or can be affected by pretreatment with antibiotics (25).

Physical and chemical changes in the blood-brain barrier allow the entry of serum proteins such as immunoglobulins and interleukins, further augmenting the antiviral process. The cell-mediated immune response is important for eliminating virus from the CNS; however, immunoglobulin also has a role in protecting the host in some viral infections. This is best illustrated by the devastating clinical course of enteroviral meningitis in agammaglobulinemic patients and in those with X-linked hyper IgM syndrome (11, 26, 27). Patients with impaired cell-mediated immunity have a higher incidence of CNS infections with certain viruses such as varicella zoster virus (VZV), measles virus, and cytomegalovirus (CMV) (4).

Clinical Manifestations

The patient's age and immune status and the viral etiology influence the clinical manifestations of viral meningitis. Patients with enteroviral meningitis often present with nonspecific symptoms such as fever (38 to 40°C) of 3 to 5 days duration, malaise, and headache (1, 11). Approximately 50% of patients have nausea or vomiting (1). While nuchal rigidity and photophobia are hallmark manifestations in meningitis, 33% of patients with viral meningitis have no evidence of meningismus (1) and less than 10% of children younger than 2 years of age develop signs of meningeal irritation. The majority of these children present with fever and irritability (28). Children may also present with seizures secondary to fever, electrolyte disturbances, or the infection itself. The clinician must have a high index of suspicion for meningitis, especially in younger patients. In the immunocompromised host, enteroviral infection presents both a diagnostic quandary and a potentially life-threatening disease. Immunocompromised patients frequently do not mount a brisk cellular immune response, so CSF analysis does not reflect inflammatory changes indicating CNS involvement.

Symptoms of meningitis (stiff neck, headache, and photophobia) occur in approximately 11% of men and 36% of women with primary HSV-2 genital infection. In one study, 5% of patients with primary HSV genital infection had severe enough meningitis to require hospitalization. All of the hospitalized patients had evidence of a lymphocytic pleocytosis upon CSF analysis (1). In another study, HSV-2 was detected in the CSF of 78% of patients with meningitis symptoms during primary genital infection. These patients also exhibited a CSF leukocytosis and increases in CSF antibody titers (29). Recurrent HSV-2 meningitis (with or without genital lesions) is seen, as is Mollaret's syndrome, but meningitis is more commonly associated with primary infection (30). Mollaret's syndrome, or benign recurrent lymphocytic meningitis, is linked to HSV and sometimes VZV infection; self-limited meningitis is characterized by CSF pleocytosis notable for large "endothelial" cells, neutrophil granulocytes, and lymphocytes. HSV meningitis may spread to the CSF along the sacral nerves. Alternatively, the virus may reach the CSF by hematogenous spread, as the virus has been cultured from the blood buffy coat layer (1). VZV, CMV, Epstein-Barr Virus (EBV), and parainfluenza virus have all been cultured or detected by PCR from the CSF of patients with meningitis (1, 12, 31). The three herpesvirus infections occur more frequently in immunocompromised patients and usually progress to involve the parenchyma.

Laboratory Findings

Initial CSF samples, while frequently suggestive of a diagnosis, lack the necessary predictive value to discriminate viral from bacterial etiology in all cases (32). Instead, epidemiologic trends, patient history, and accompanying laboratory information are important adjuncts in determining the etiology of meningitis. CSF in patients with viral meningitis typically exhibits a pleocytosis with 10 to 500 leukocytes mm^3 and a slightly elevated protein level (< 100mg/dl). The glucose level in the CSF is typically greater than 40% of a simultaneously drawn serum sample. Tremendous variation in CSF formulas exists, however, with significant overlap between viral and bacterial CSF laboratory findings (32). In a retrospective review of over 400 patients with acute viral or bacterial meningitis performed before the *Haemophilus influenza B* conjugate vaccine was available, investigators found that approximately 20% of the CSF samples that grew bacteria exhibited a CSF pleocytosis of less than 250 WBC/mm^3 (1). Fifteen percent of the patients with bacterial meningitis had CSF lymphocytosis, while 40% of the patients with viral meningitis had a predominance of polymorphonuclear cells. Some investigators recommend repeating the lumbar puncture 6 to 12 hours later, because the CSF profile of patients with viral meningitis will shift from polymorphonuclear to lymphocytic pleocytosis over this period (1). However, in one study performed during an echovirus epidemic, eight of nine children with presumed enteroviral meningitis failed to develop CSF lymphocyte predominance when a lumbar puncture was repeated 5 to 8 hours later (32). A retrospective study found that 51% of patients demonstrated a CSF polymorphonuclear predominance 1 day after

symptom onset. Of note, the investigators in this study were unable to confirm the etiology of meningitis in most cases because this was a retrospective study (32). Other investigators have confirmed that the change in the lymphocytic CSF profile occurs 18 to 36 hours after illness onset (33). A multifactorial method examining the CSF profile, peripheral blood profile, and history of seizures to develop a bacterial meningitis score has also been investigated to differentiate bacterial from viral meningitis. This provides improved sensitivity but still failed to detect all cases of bacterial meningitis, including some cases in infants (34). With the increasing use of highly sensitive and specific nucleic-acid diagnostic techniques, a viral etiology for meningitis can often be established within 24 to 36 hours, thereby limiting the duration of hospitalization, antibiotic use, and additional diagnostic procedures (7, 9, 13).

Etiologic Diagnosis

Historically, the techniques for identifying viral meningitis were insensitive and often impractical, with an agent identified in only 25% to 67% of presumed CNS infections (1, 12, 35). The diagnosis of viral meningitis relied on viral culture, and CSF viral culture rates differ based on etiology (1).

A synopsis of viral detection techniques for different viruses is presented in Tables 1 and 2 (4). The rapidity and sensitivity of enterovirus detection, as well as for many other viral infections, has improved with the advent of nucleic acid amplification techniques such as RT-PCR (9, 11). Demonstration of viral nucleic acid in the CSF of patients with symptoms of meningitis has replaced viral isolation and serologic diagnosis for acute management, although one of the advantages of viral culture is the ability to identify enterovirus serotypes for epidemiologic studies (9, 11). As with any RT-PCR-based technique, nucleic acid contamination of the laboratory area is a concern and results must always be interpreted within the clinical context, although use of deoxyuridine triphosphatase (dUTP) and modifications to real-time PCR methods have reduced this risk (36, 37).

In the past, serologic testing confirmed the clinical suspicion of an arboviral infection. While many of these viruses can be cultured from CSF during the early stages of infection, serologic testing has very limited availability and little utility in acute management. The use of RT-PCR for diagnosis of arbovirus infections in the CNS has met with mixed results because of the diverse viral etiologies of arboviral infection. The development of specific primers that can hybridize across multiple viral families (*Alphaviradae*, *Flaviviradae*, *Bunyaviradae*) has been difficult. Currently there is an emphasis on the development of improved "universal group primers" to perform an initial group screening followed by RT-PCR using higher specificity primers as a second viral diagnostic test (38). Many laboratories have chosen instead to concentrate efforts on establishing diagnostic studies for more common regional viral etiologies as a more cost-effective method for diagnosis and patient management (7).

Differential Diagnosis

Unusual but treatable infections should always be considered and investigated in patients with CSF pleocytosis and negative conventional bacterial cultures. Spirochetes (*Treponema*, *Borrelia*, *Leptospira*), mycoplasma, bartonella, and mycobacteria can produce pleocytosis that has both negative Gram stain and bacterial cultures. Fastidious bacteria (*Listeria*) may fail to grow in culture and occasionally produce a mononuclear pleocytosis similar to viral meningitis. This is of particular concern in infants, elderly, and immunocompromised patients. Some bacteria, while not directly infecting the CNS, can release toxins that create a change in the level of consciousness, specifically *Staphylococcus aureus* and *Streptococcus pyogenes* exotoxin-mediated toxic shock syndrome. Frequently, children with streptococcal throat infections present with "neck stiffness" secondary to localized pharyngeal inflammation and tender anterior cervical lymphadenopathy. Parameningeal infections, especially from infected sinuses, produce CNS symptoms and pleocytosis presenting with nuchal rigidity, focal neurologic changes, and altered mental status. Similarly, partially treated bacterial infections can have CSF findings resembling viral meningitis. Questions regarding history of self-medication with leftover antibiotics should be included in a review of systems.

Fungal and parasitic infections can produce both meningitis and parenchymal CNS infections. Coccidiomycosis and *Cryptococcus*, the leading causes of fungal meningitis (1), characteristically produce meningitis rather than any focal CNS disease. Fungal infections such as candidiasis, aspergillosis, histoplasmosis, and blastomycosis most frequently cause focal parenchymal disease when infecting the CNS. These fungi are frequently in the differential diagnosis for an immunocompromised host with CNS disease but can cause disease in select patient populations (e.g., premature infants and patients with CNS trauma and diabetes mellitus). Parasites such as *Naeglaeria fowleri* or *Balamuthia mandrillaris* produce meningoencephalitis with purulent CSF findings (39, 40). A history of recent summertime swimming in a stagnant pond or recent travel in Central or South America should raise suspicion for these infections.

Noninfectious processes that can produce true aseptic meningitis include hematologic malignancies, medications (especially immunomodulatory, nonsteroidal antiinflammatory, and trimethoprim/sulfa medications), autoimmune diseases, and foreign material and proteins. Leukemia produces CSF pleocytosis with cancerous cells and occurs most frequently with acute lymphocytic leukemia, although subarachnoid involvement can also occur in acute myelogenous leukemia. Immunomodulatory drugs such as intravenous immunoglobulin or antilymphocyte globulin (OKT-3) also cause aseptic meningitis. Of the medications associated with meningitis, nonsteroidal antiinflammatory agents, sulfa-containing drugs, and cytosine arabinoside are the most common offenders. Drug-induced aseptic meningitis frequently occurs in patients with underlying connective tissue or rheumatologic diseases. A patient with drug-induced aseptic meningitis warrants investigation for a possible underlying autoimmune disease 41). Epithelial or endothelial cysts can rupture and spill their contents (keratin, protein), producing a brisk inflammatory response that mimics acute viral meningitis.

Treatment and Prognosis

The fundamental principle of therapy for viral meningitis lies in the identification of potentially treatable diseases. Until recently, no therapy existed for most cases of viral meningitis. Efforts instead focused on preventive strategies (largely through vaccination) as well as identification of treatable nonviral etiologies of meningitis. The clinician must also anticipate and treat the complications of viral CNS disease (seizures, syndrome of inappropriate antidiuretic hormone secretion [SIADH], hydrocephalus, increased intracranial pressure). Supportive therapy includes hydration, antipyretics, and analgesics.

In the normal host, viral meningitis is a relatively benign self-limited disease. A prospective study in children less than 2 years of age, for example, found that even in the 9% of children who develop evidence of acute neurologic disease (complex seizures, increased intracerebral pressure, or coma) their long-term prognosis is excellent. During long-term follow up (42 months of age), children with acute CNS complications performed neurodevelopmental tasks and achieved developmental milestones as well as children with an uncomplicated course (28). An overview of the approach to a patient with suspected viral CNS disease is presented in a later section.

Antibody preparations and the antiviral agent pleconaril have shown activity against enterovirus in small series (42, 43) and animal studies (44–46). However, randomized controlled trials have not supported their routine use in enterovirus meningitis (47, 48). The literature contains case reports of immunoglobulin preparations improving outcome in agammaglobulinemic patients with enteroviral meningitis. However, immunoglobulin use in these patients does not eliminate the virus from the CSF or prevent chronic enteroviral meningitis (1). Enteroviral infections in neonates frequently produce overwhelming viremia and CNS disease. Ten percent of neonates with systemic enteroviral infections die, and as many as 76% are left with permanent sequelae. Standard intravenous immunoglobulin does not provide clinical benefit for neonates with severe life-threatening enteroviral infection (42). While the role of antibody\ies in immunocompromised patients with life-threatening enteroviral infections remains debatable, there are currently no data supporting the use of immunoglobulin preparations for non-life-threatening infections in the normal host.

Specific antiviral agents are available for meningitis of several other etiologies. Although no definitive clinical trials have been conducted, most experts recommend the use of IV acyclovir for HSV meningitis, as it decreases the duration of primary herpes disease and may limit meningeal involvement (49). Recurrent HSV-2 meningitis is rare, and recently a single case of meningitis associated with HSV-1 reactivation was reported. At this time there are no data on benefits of antiviral treatment or suppressive therapy for recurrent HSV CNS disease (1, 50). Effective antiviral therapy exists for VZV infections of the CNS and should be instituted in these patients (51, 52). For CMV CNS infection in the immunocompromised host, therapy is problematic and should be tailored based upon the clinical likelihood of infection.

VIRAL ENCEPHALITIS

Epidemiology and Prevalence

Similar to the case with viral meningitis, passive reporting systems underestimate the incidence of viral encephalitis (1). An estimated 20,000 cases of encephalitis occur each year in the United States; however, the CDC received only 740 (0.3/100,000) to 1340 (0.54/100,000) annual reports of persons with encephalitis from 1990 to 1994 (1). A review of the cases in Olmsted County, Minnesota, from 1950 to 1980 found the incidence of viral encephalitis was at least twice as frequent as that reported by the CDC (6). A prospective study in Finland demonstrated similar results, with the incidence of encephalitis being 10.5/100,000 (53).

Although nucleic acid-based diagnostic testing has enhanced the detection of many viruses, a viral etiology is not found in the majority (83%) of cases of encephalitis (12). While the etiology of encephalitis has changed with alterations in the viral reservoirs in North America, the overall death rates from encephalitis have not changed since the late 1970s to 1980s (54). HSV CNS infections occur without seasonal variation, affect all ages, and constitute the majority of fatal cases of endemic encephalitis in the United States (1). Arboviruses, a group of over 500 arthropod-transmitted RNA viruses, are the leading cause of encephalitis worldwide and in the United States (4). Arboviral infections occur in epidemics and show a seasonal predilection, reflecting the prevalence of the transmitting vector (1). Asymptomatic infections vastly outnumber symptomatic infections. Patients with disease may develop a mild systemic febrile illness or viral meningitis (1). Encephalitis occurs in a minority of people with arboviral infections, but the case-fatality rate varies extremely, from 5% to 70%, depending upon viral etiology and age of the patient. Neuroinvasive WNV infections now far outnumber other arboviral causes of encephalitis in the United States (55), although it is unknown if this is because of improved testing and more active surveillance. Historically, LaCrosse encephalitis has been the most commonly reported arboviral disease in the United States, while St. Louis encephalitis is the most frequent cause of epidemic encephalitis (1, 56). Characteristically, Eastern equine encephalitis (EEE) and most arboviral infections occur in the late summer following amplification of the virus due to peak mosquito activity (1). In warm climates the clinician must have a high index of suspicion for insect-borne diseases.

Japanese B encephalitis and rabies constitute the majority of cases of documented encephalitis cases outside of North America. Japanese encephalitis virus, a mosquito-transmitted member of the flavivirus genus, occurs throughout Asia and causes epidemics in China despite routine immunization (1, 57). In warmer locations, the virus is endemic (1, 58). The disease typically affects children, although adults with no history of exposure to the virus are also susceptible (1). As with the other arboviral infections, asymptomatic infections occur more frequently than symptomatic infections. However, the disease has a high case-fatality rate and leaves half of the survivors with a significant degree of neurologic morbidity (1).

Rabies virus remains endemic in much of the world. In the United States human cases have decreased over the last decades to 1 to 3 cases per year as a result of the immunization of domesticated animals. Bats are increasingly recognized as a source of infection. In one study, 15% percent of bats tested carried rabies virus (59). Since 1990, bat-associated variants of the virus have accounted for 24 of the 32 cases in the United States. In most cases there was no evidence of a bite, although in half of the cases direct contact (handling of the bats) was documented (60). In areas outside the United States, human cases of rabies encephalitis number in the thousands and are caused by unvaccinated domestic animals, principally dogs, developing infection following contact with infected wild animals and exposure to bat guano (61).

Postinfectious encephalitis, an acute demyelinating process also referred to as acute disseminated encephalomyelitis (ADEM) or autoimmune encephalitis, accounts for approximately 100 to 200 additional cases of encephalitis annually in the United States (1, 62). The disease historically was responsible for approximately one third of the encephalitis cases in the United States and was associated with preceding measles, mumps, and other exanthematous viral infections (1, 62). Postinfectious encephalitis in the United States is now associated with antecedent upper respiratory virus (notably influenza virus) and varicella infections (1).

Measles continues to be the leading cause of both acute encephalitis, in the absence of vaccination, and post-infectious encephalitis worldwide and complicates 1 of every 1000 measles infections (1). Autoimmune CNS damage and an ADEM process has also been reported following cases of acute encephalitis and in paraneoplastic syndromes. Some patients develop N-methyl-D-aspartate (NMDA) autoantibodies with their paraneoplastic syndrome 1 to 4 weeks following acute encephalitis (34, 63). Recent studies suggest that antigenic variation in the N-terminal domain of the NMDA receptor may predispose some patients to the autoimmune encephalitis.(35, 64).

The slow infections of the CNS or transmissible spongiform encephalopathies (TSE) occur sporadically worldwide. The prototypical TSE is Creutzfeld-Jacob disease (CJD); occurs at high rates within families and has an estimated incidence of 0.5 to 1.5 cases per million populations (5). In 1986, cases of a TSE in cattle termed bovine spongiform encephalopathy (BSE) were reported in the United Kingdom. In addition to affecting other livestock throughout Europe that were fed supplements containing meat and bone meal, cross-species transmission of BSE has been documented, leading to a ban in the use of bovine offal in fertilizers, pet food, and other animal feed (5). A decrease in recognized cases of BSE has occurred since the institution of these restrictions. Concomitant with the increased cases of BSE in Europe, an increase in cases of an atypical Creutzfeldt-Jacob disease also occurred, suggesting animal-to-human transmission. The report of atypical CJD (unique clinical and histopathologic findings) affecting young adults (an age at which CJD rarely has been diagnosed) and a characteristic methionine at the polymorphic codon 129 led to the designation of a new disease, variant Creutzfeldt-Jacob disease (vCJD). As of 2006, a total of 160 cases of vCJD were diagnosed in the United Kingdom (UK) and 28 cases outside of the UK (65).

Pathogenesis

The pathogenesis of encephalitis requires that viruses reach the CNS by hematogenous or neuronal spread. Similar to meningitis, viruses most frequently access the CNS after a high-titer secondary viremia and cell-free or cell-associated CNS entry (4). Other than direct entry via cerebral vessels, the virus can initially infect the meninges and CSF and then enter the parenchyma across either ependymal cells or the pial linings. Viruses exhibit differences in neurotropism and neurovirulence; receptor requirements are one determinant of viral neurotropism. For example, enteroviruses with similar receptors produce very different diseases. Five coxsackie B viruses (types B1 to B5) readily produce CNS infections while type B6 rarely produces neurologic infection (4, 66). Viral genes have been discovered that influence the neurovirulence of HSV-1 (67). Mutant HSV-1 viruses with either $\gamma_1$34.5 gene deletions or stop codons inserted into the gene have a decreased ability to cause encephalitis and death following intracerebral inoculation in mice as compared to wild-type virus (67, 68).

In addition to viral factors, host physiology is also important in determining the extent and location of viral CNS disease. Age, sex, and genetic differences among hosts influence viral infections and clinical course (11, 69). Host age influences the clinical manifestations and sequelae of viral infections. For example, sindbis virus infection produces lethal encephalitis in newborn mice, while weanling mice experience persistent but nonfatal encephalitis. The reason for the difference in outcome is twofold. Mature neurons resist viral-induced apoptosis and older mice have an improved antibody response, thus limiting viral replication (69). Variations in macrophage function among individuals can result in clinically distinct infections and disease. Moreover, macrophage-antigen response can change with age and is important in limiting spread of infection within a patient (1). In addition to age, physical activity may be another important host factor that determines the severity of infection. Exercise has been associated with increased risk for paralytic poliomyelitis and may result in an increased incidence of enteroviral myocarditis and aseptic meningitis (1). Increasingly, host differences are recognized as equally important determinants of disease at the cellular and molecular levels.

Historically, the peripheral neural pathway was considered the only pathway of viral neurologic infection, although contemporary data demonstrate that the circulatory system is more commonly implicated (1). Herpes simplex virus and rabies are examples of viruses that infect the CNS by neuronal spread. Sensory and motor neurons contain transport systems that carry materials along the axon to (retrograde) and from (anterograde) the nucleus. Peripheral or cranial nerves provide access to the CNS and shield the virus from immune regulation. Lastly, olfactory nerve transport to the CNS is a logical explanation for HSV infections of the brain when the nasopharynx is a site of viral replication.

Rabies classically infects through the myoneural route and provides a prototype for peripheral neuronal spread (4, 60). Rabies virus replicates locally in the soft tissue following a bit by a rabid animal. After primary replication, the virus enters the peripheral nerves by binding to acetylcholine receptors. Once in the muscle the virus buds from the plasma membrane, crosses myoneural spindles, or enters across the motor end plate (4). The virus travels by anterograde and retrograde axonal transport to infect neurons in the brainstem and limbic system. Eventually the virus spreads from the diencephalic and hippocampal structure to the remainder of the brain, killing the animal (4).

Virus also infects the CNS through cranial nerves. Animal studies have shown that HSV can infect the brain through the olfactory system as well as the trigeminal nerve (4). Early HSV encephalitis damages the inferomedial temporal lobe that contains direct connections with the olfactory bulb (1). The route of human HSV infections, however, is less clear. Despite data supporting olfactory and trigeminal spread of virus to the CNS, definitive proof in humans is lacking. The association of viral latency in the trigeminal ganglia, the relative infrequency of herpes simplex encephalitis (HSE), and the confusing data regarding encephalitis from HSV reactivation suggest that the pathogenesis is more complex than described above (49).

In patients with acute encephalitis, the parenchyma exhibits neuronophagia and cells containing viral nucleic acids or antigens. The pathologic findings are unique for different viruses and reflect differences in pathogenesis and virulence. In the case of typical HSV encephalitis, hemorrhagic necrosis occurs in the inferomedial temporal lobe with evidence of perivascular cuffing, lymphocytic infiltration, and neuronophagia (4). Pathological specimens in animals with rabies encephalitis have microglial proliferation, perivascular infiltrates, and neuronal destruction. The location of the pathological findings can be limited to the brainstem areas (dumb rabies) or the diencephalic, hippocampal, and hypothalamic areas (furious rabies) based on the immune response mounted against the infection (4).

Some viruses do not directly infect the CNS but produce immune system changes that result in parenchymal damage.

Patients with postinfectious encephalitis (ADEM) exhibit focal neurologic deficits and altered consciousness temporally associated with a recent (1 to 2 week) viral infection or immunization (62). Pathologic specimens, while they show evidence of demyelination by histologic or radiographic analysis, do not show evidence of viral infection in the CNS in culture or antigen tests. Patients with postinfectious encephalitis have subtle differences in their immune systems, and some authors have proposed an autoimmune reaction as the pathogenic mechanism of disease (1). Postinfectious encephalitis occurs most commonly following measles, VZV, mumps, influenza, and parainfluenza infections. With immunization the incidence of postinfectious encephalitis has decreased in the United States; however, measles continues to be the leading cause of postinfectious encephalitis worldwide (1). As noted above, in addition to the postinfectious process, patients with paraneoplastic syndrome and autoantibodies to NMDA autoantibodies have also recently been described (34). Recent studies suggest that antigenic variation in the N-terminal domain of the NMDA receptor may predispose these patients to autoimmune encephalitis (35). A subset of patients developed anti-NMDA autoantibodies 1 to 4 weeks following HSE that leads to recurrent immune-mediated encephalitis, complicating their recovery.

The TSEs are noninflammatory CNS diseases involving the accumulation of an abnormal form of a normal glycoprotein, the prion protein (PrP) (1). These encephalopathies differ in mode of transmission. While most of the TSEs are experimentally transmissible by direct inoculation in the CNS, this mode rarely occurs except for iatrogenic transmissions (70). The scrapie agent spreads by contact and lateral transmission. There is no evidence for lateral transmission for BSE or vCJD and all cases appear to have occurred following parenteral or ingestion of affected materials. The transmissible agents remain infectious after treatments that would normally inactivate viruses or nucleic acids (detergent formalin, ionizing radiation, nucleases) (1). Most of the experimental work on TSEs has involved analysis of the scrapie agent. The current working model is that posttranslational alteration of the normally α-helical form of the PrP protein results in a protease resistant β-pleated sheet structure that accumulates in neurons leading to progressive dysfunction, cell death, and subsequent astrocytosis. In studies on the scrapie agent and vCJD, gastrointestinal tract involvement with infection of abdominal lymph nodes occurs first, followed by hematogenous spread through the reticuloendothelial system and brain involvement a year or more later (1, 71). Experimental subcutaneous inoculation in mice and goats also led to local lymph node involvement, followed by splenic spread and then CNS involvement. Cases of vCJD through blood transfusion have also occurred (72). Based upon animal studies, there is an equal distribution of the agent associated with leukocytes and free in the plasma, with negligible levels associated with the red blood cells and platelets (73).

Clinical Manifestations

Patients with encephalitis have clinical and laboratory manifestations of parenchymal disease; however, infection rarely involves only the brain parenchyma. Some viruses (rabies, herpes B virus) produce encephalitis without significant meningeal involvement; however, most patients with encephalitis have concomitant meningitis. Most patients also have a prodromal illness with myalgias, fever, and anorexia, reflecting the systemic viremia. Neurologic symptoms can range from fever, headache, and subtle neurologic deficits or change in level of consciousness to severe disease with seizures, behavioral changes, focal neurologic deficits, and coma (4). Clinical manifestations reflect the location and degree of parenchymal involvement and differ based on viral etiology. For example, HSE infects the inferomedial frontal area of the cortex, resulting in focal seizures, personality changes, and aphasia. These symptoms reflect the neuroanatomical location of infection with inflammation near the internal capsule and limbic and Broca's regions (4). Paraesthesias near the location of the animal bite and changes in behavior correlate temporally with the axoplasmic transport of rabies and the viral infection of the brainstem and hippocampal region (1, 74). Rabies has a predilection for the limbic system, thereby producing personality changes. The damage spares cortical regions during this phase, so that humans may vacillate between periods of calm, normal activity and short episodes of rage and disorientation (4). Alternatively, Japanese encephalitis virus initially produces a systemic illness with fever, malaise, and anorexia, followed by photophobia, vomiting, headache, and changes in brainstem function. Most children die from respiratory failure and frequently have evidence of cardiac and respiratory instability, reflecting Viremic spread via the vertebral vessels and infection of brainstem nuclei (4). Other patients have evidence of multifocal CNS disease involving the basal ganglia, thalamus, and lower cortex and develop tremors, dystonia, and parkinsonian symptoms (4).

Encephalitis, unlike meningitis, often has high mortality and complication rates but these differ based on the viral etiology and host factors (91, 75). For example, St. Louis encephalitis virus has an overall case mortality of 10%; the rate is only 2% in children but increases to 20% in the elderly (76). Other viruses like Western equine and Eastern equine encephalitis produce higher mortality and morbidity in children than in adults (76).

The TSEs are slowly progressing diseases with long incubation periods. Sporadic CJD occurs between the ages of 50 and 70 years of age and is characterized by dementia, tremors, and, more rarely, abnormal movements and ataxia. Unlike sporadic CJG, vCJD disease affects young adults and adolescents; it produces cerebellar ataxia and sensory involvement (dysesthesias) with florid amyloid plaques detected in the brain on autopsy. Neurologic deterioration progresses relentlessly, and most patients die less than a year after onset of their neurologic manifestations.

Laboratory Findings and Diagnosis

Establishing a diagnosis requires a meticulous history, knowledge of epidemiologic factors, detailed microbiologic studies, and a systematic evaluation of other possible treatable diseases. In the past, investigators failed 50% to 75% of the time to identify an etiology for encephalitis depending on the study and diagnostic tests used (1). CSF pleocytosis usually occurs in encephalitis but is not necessary for the diagnosis. White blood cell counts in CSF typically number in the 10's to 100's in viral encephalitis (1). Cerebrospinal glucose levels are usually normal although some viral etiologies (e.g., EEE) produce CSF parameters consistent with acute bacterial meningitis (1). Some viruses (e.g., HSV) produce a hemorrhagic necrosis and the CSF exhibits this by moderately high protein levels and evidence of red blood cells. Supratentorial and cerebellar tumors can produce increased intracranial pressure and can mimic encephalitis. A careful fundoscopic exam and appropriate radiographic imaging should be performed prior to obtaining CSF to rule out

any evidence of papilledema and increased intracranial pressure.

Unlike meningitis, encephalitis often requires additional laboratory and radiologic tests to establish the diagnosis. Historically, the standard for diagnosis was brain biopsy and viral isolation. For many viruses (e.g., HSV, enterovirus, EBV, VZV, John Cunningham [JC] virus, HHV6, tick-borne encephalitis [TBV]) detection of viral nucleic acids by PCR or RT-PCR from the CSF has replaced culture and brain biopsy as the standard for diagnosing encephalitis (1, 77, 78). Computerized tomography (CT scan) and magnetic resonance imaging (MRI) are needed to look for focal encephalitic changes, parenchymal necrosis or bleeding, demyelination, and mass lesions. The increased sensitivity of MRI to alterations in brain water content and the lack of bone artifacts make this the neuroradiologic modality of choice for CNS infections (79, 80). MRI and especially diffusion-weighted imaging detect parenchymal changes earlier than CT scan and better defines the extent of a lesion (81). Furthermore, MRI is more sensitive for detecting evidence of demyelinating lesions in the periventricular and deep white matter, thus enabling differentiation of parainfectious from acute viral encephalitis. Patients with viral encephalitis frequently have diffuse or focal epileptiform discharges with background slowing on electroencephalogram (EEG) (4). These EEG changes precede CT scan evidence of encephalitis and provide a sensitive although nonspecific diagnostic test. EEG changes in the temporal lobe area strongly support the diagnosis of HSE; however, the absence of these changes does not rule out HSE.

Historically, patients with viral encephalitis required a battery of different diagnostic tests. HSV encephalitis, for example, could be diagnosed acutely by brain biopsy and viral culture or retrospectively by CSF antibody and convalescent serologic tests (4). The diagnosis of enterovirus meningitis previously required acute virus isolation from the throat or rectum acutely or retrospective serologic studies. Molecular techniques are not routinely used for the diagnosis of most viral CNS infections (4, 82). Primers also exist for the detection of certain arboviral encephalitides (California encephalitis group, Japanese encephalitis, West Nile, St. Louis encephalitis, dengue fever serotypes 1–4, and yellow fever viruses); however the development of universal arboviral primers has been more difficult (1, 4, 38). The successful detection of viral nucleic acids in the CSF is influenced by the duration, extent, and etiology of disease. The laboratory test is relatively rapid, has high sensitivity, and provides a less invasive means to diagnose encephalitis. For example, only 4% of CSF cultures are positive in patients with sporadic HSE; in patients with biopsy-proven HSE, CSF PCR has a sensitivity of greater than 95% and a specificity approaching 100% (83). Interestingly, in the three cases where the CSF PCR was positive but the brain biopsy negative, biopsy samples had been improperly prepared prior to viral culture or the biopsy site was suboptimal (1). Recently, efforts have focused on correlating viral nucleic acid copy as an indicator of virus quantity to predict clinical outcome (84).

The clinical diagnosis of a TSE is supported by detection of characteristic EEG changes (periodic sharp and slow wave complexes), presence of 14–3-3 protein in the CSF, and characteristic MRI findings (increased signal in the basal ganglia in vCJD or evidence of increased signal in the posterior pulvinar in vCJD) (85). Most laboratory tests are of little value in the diagnosis in humans. CSF examination shows normal values or slightly elevated protein levels. The EEG in classic CJD reveals generalized slowing early in the disease and biphasic or triphasic peaks late in the disease with the onset of myoclonus. MRI changes late in the illness reveal global atrophy with hyperintense signals from the basal ganglia (5). Diffusion-weighted imaging and fluid attenuation inversion recovery (FLAIR) remain the most reliable and sensitive imaging techniques for CJD (85, 86). Histopathologic examination of the brain using a specific antibody to the PrP-res protein confirms the disease. In addition, evidence of gliosis, neuronal loss, and spongiform changes support the diagnosis. In cases of vCJD, characteristic microscopic amyloid plaques (so-called florid plaques) define the disease. The florid plaques are not seen in other TSEs and consist of flower-like amyloid deposits surrounded by vacuolar halos. The detection of PrP-res in the tonsillar tissue by immunohistochemical staining is also strongly supportive of a vCJD diagnosis (5).

Differential Diagnosis

Identifying treatable disease expeditiously is a priority in patients presenting with neurologic changes. In patients with suspected HSE undergoing brain biopsy for confirmation, alternative diagnoses are frequently found. Of 432 patients, only 45% had biopsy-confirmed HSE and 22% had another etiology established by brain biopsy (3). Of these, 40% had a treatable disease (9% of the biopsy group) including bacterial abscess, tuberculosis, fungal infection, tumor, subdural hematoma, or autoimmune disease. The majority of the remaining 60% identifiable but non-treatable causes for encephalitis were of viral etiology. Disease in a third group of 142 patients (33%) went undiagnosed even after brain-biopsy and the conventional diagnostic tests.

Pathologic processes in the CNS have limited clinical expressions and thus often produce similar signs and symptoms (4). Other causes of encephalitis are presented in Table 3. Mass lesions in the CNS (tumor, abscess, or blood) can cause focal neurologic changes, fever, and seizures, similar to encephalitis. Metabolic (hypoglycemia, uremia, inborn errors of metabolism) and toxin-mediated disorders (ingestion, tick-related paralysis, or Reye syndrome) can cause decreased consciousness, seizures, and background slowing on EEG. Limbic encephalitis can produce protracted encephalitis and is caused by paraneoplastic phenomena. Furthermore, treatable infectious causes of encephalitis must be vigorously investigated. Mycoplasma produces demyelinating brainstem encephalitis in approximately 0.1% of infections.

Prevention

Prevention of the initiating viral infection remains the best means to reduce risk of viral encephalitis. Live attenuated vaccines against measles, mumps, and rubella have resulted in a dramatic decrease in the incidence of encephalitis in industrialized countries. Measles continues to be the leading cause of postinfectious encephalitis in developing countries, however, and complicates 1 of every 1000 measles infections (1). Widespread polio vaccination has eradicated the disease at present from the Western Hemisphere and most other countries. Vaccines exist for some arboviral infections and, of course, rabies. Vaccination against Japanese encephalitis virus has reduced the incidence of encephalitis in Asia but cases still occur annually (57, 87, 88).

Vaccination and antiviral chemoprophylaxis are either not available or cost-effective for preventing many viral infections. For example, vector avoidance, the use of mosquito deterrents, and mosquito abatement programs are less costly strategies for preventing arboviral encephalitides in

TABLE 3 Differential diagnosis for encephalitis and meningitis

Infectious
Bacterial
Common organisms (*S. pneumoniae, S. agalactiae, N. meningitis, H. influenza*)
Complex bacteria (*Mycobacterium, Actinomyces, Nocardia*)
Spirochetes (*Treponema, Borrelia, Leptospira*)
Cell associated (*Rickettsia, Ehrlichia, Mycoplasma*) *Brucella, Listeria, Bartonella*
Partially treated bacterial infection
Abscess (brain, parameningeal)
Bacteria-produced toxin
Fungal
Blastomyces, Candida, Histoplasma, Coccidioides, Aspergillus, Sporothrix, Zygomycetes, other)
Parasites and Protozoan
Toxoplasma, Taenia solium, Echinococcus, Strongyloides, Schistosoma, Acanthamoeba, Naegleria fowleri, Balamuthia mandrillaris, Trypanosoma, Plasmodium
Postinfectious
Guillain-Barre' Syndrome
Brain stem encephalitis
Miller-Fisher Syndrome
Acute disseminated encephalomyelitis (ADEM) postviral (varicella zoster, measles, parainfluenza, influenza, respiratory syncitial virus), unknown
Limbic encephalitis
Paraneoplastic syndrome

the United States (1, 89). Pre- and immediate postexposure prophylaxis are the only ways known to prevent death in rabies-exposed individuals (90). Individuals exposed to rabies require vigorous cleansing of the wound, postexposure vaccination, and direct administration of rabies hyperimmunoglobulin at the site of the animal bite. Individuals with frequent contact with potentially rabid animals (veterinarians, animal control staff, workers in rabies laboratories, and travelers to rabies-endemic areas) should receive preexposure vaccination. The FDA has implemented guidelines eliminating whole blood or blood components prepared from individuals who later developed CJD or vCJD to reduce the potential exposure to TSE agents in the blood supply. While four cases of transfusion associated vCJD have been reported, the risk associated with packed red blood cells (PRBC) and platelet transfusion is less than that for patients receiving large amounts of whole blood (73). Changes in agricultural practices in Europe, testing for affected cattle, and bans on infected cattle have been associated with decline in cases of vCJD. In North America no cases of vCJD have been reported and the Department of agriculture has programs in place to monitor for TSEs in livestock (91).

Treatment

Patients with encephalitis require treatment tailored to the etiology and clinical situation. Currently few antiviral medications are available to treat CNS infections. Antiviral therapy exists for HSV-1, HSV-2, VZV, CMV, and HIV. The introduction of acyclovir has resulted in a sharp decline in mortality and morbidity from HSV infections; neonatal mortality from disseminated HSV disease and HSE has declined from 70% to 40% and antiviral treatment also reduces the severity of neurologic impairment (1). Varicella immunoglobulin (VZIG) and acyclovir have reduced the complications from primary VZV infection and zoster in neonates and immunocompromised patients. Although controlled trials have not evaluated the efficacy of acyclovir in VZV encephalitis, the medication is routinely used to treat this complication (1, 92). Ganciclovir and foscarnet are used for the treatment of CMV encephalitis although controlled clinical trials have not confirmed the efficacy of this treatment. Antiretroviral therapy appears to decrease the frequency and severity of HIV CNS disease, but studies have not determined if this is because of a direct reduction in HIV viral activity in the CNS or a secondary effect as a result of improved immune function and decreased opportunistic infections affecting the CNS (93).

In cases of postinfectious encephalitis or ADEM, no randomized controlled trial has confirmed the benefit of immunomodulatory drugs. In practice clinicians often treat ADEM with different immunomodulators in an attempt to limit T-cell-mediated destruction of the CNS (62). It must be emphasized, however, that immunomodulatory therapy is based on isolated case reports and series. Cinical failures and iatrogenic morbidity from a therapeutic modality are rarely ever reported.

Approach to Patients with Viral CNS Disease

The approach to a patient with a presumed CNS viral infection must be tailored to the severity and distribution of neurologic involvement. The degree of diagnostic as well as therapeutic intervention differs based on the type of CNS disease. The examination and radiographic and laboratory studies available for establishing a diagnosis must be prioritized based on the likely etiology and the stability of the patient. For example, a patient with photophobia and nuchal rigidity but a nonfocal neurologic examination does not require invasive intracranial pressure monitoring as would a patient with encephalitis and evidence of increased intracranial pressure. After establishing the degree of CNS disease by history and physical exam and stabilizing the patient (airway, breathing, and circulation), the clinician next must ascertain a diagnosis.

Treatable causes of CNS dysfunction require rapid evaluation and intervention in an effort to prevent further or permanent CNS damage. Potentially treatable diseases (e.g., HSE, VZV, fungal infections, partially treated bacterial meningitis, tuberculous meningitis, parameningeal infection, mycoplasma, and fastidious bacterial infections) can mimic viral CNS disease and should be vigorously investigated before attributing the illness to a viral etiology for which no specific antiviral is available. The same logic applies to treatable viral infections and noninfectious etiologies. After establishing a presumptive diagnosis and instituting therapy, the clinician must vigilantly anticipate and treat complications associated with the viral CNS disease or the therapeutic interventions. Seizures secondary to direct viral CNS damage, inflammatory vasculitis, and electrolyte changes require anticonvulsant therapy with benzodiazepams, phenytoin, and barbiturates (1). Patients with cerebral edema may require intracranial pressure monitoring and hyperventilation, osmotic therapy, and CSF removal in an attempt to maintain cerebral pressures (1). The ultimate goal of intracranial pressure monitoring is to maintain adequate cerebral perfusion. While a physician

struggles to maintain an adequate intravascular blood volume, intracranial pressures can rise to dangerous levels as capillary leaks complicate the patient's course. The risks of increased intracranial pressure from aggressive fluid resuscitation or the syndrome of inappropriate antidiuretic hormone release necessitates fastidious fluid management and frequent electrolyte monitoring. Cardiac arrhythmias can also develop in patients with encephalitis secondary to electrolyte changes or brainstem damage. Cardiac and respiratory arrest can occur early in disease; therefore, equipment for intubation and cardioversion should be readily available for a patient with encephalitis. In addition to the direct damage the virus can cause in the CNS, certain viruses can also produce systemic damage that complicates the management of the CNS disease. Patients can develop overwhelming hepatitis, pneumonitis, disseminated intravascular coagulation, and shock. Patients in coma from encephalitis can recover after long periods of unconsciousness. The physician should strive to limit the amount of iatrogenic damage and vigorously support the patient during the acute phase of the illness.

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Gastrointestinal Syndromes

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4

Gastroenteritis is a major cause of morbidity and mortality in humans, and viruses are important causes of this disease. While many viruses have been associated with diarrhea in humans, we know most about rotavirus because the methods used to detect it are best developed. Rotavirus remains the most important cause of severe diarrhea in children worldwide. Implementation of effective vaccines has resulted in a substantial reduction of the rotavirus disease burden. As rotavirus incidence drops in countries with mature vaccination programs, norovirus is increasingly being recognized as a major cause of pediatric diarrhea.

The gastroenteritis viruses fall into two distinct epidemiologic groups: those that cause common childhood diarrhea in early life—rotavirus, adenovirus, caliciviruses (norovirus and sapovirus), and astrovirus—and those responsible for epidemic disease, primarily noroviruses but also astrovirus and group B rotavirus.

All of these viruses cause a clinical syndrome of diarrhea and vomiting that is generally similar, extraintestinal manifestations of disease are rare. Some groups of people are at particularly high risk for disease with these agents by virtue of their age (the young and the old), their extent of exposure, or their host susceptibility.

The primary treatment of all these diseases is fluid and electrolyte replacement. Prevention of the main childhood disease, rotavirus diarrhea, is based on widely used live-attenuated oral vaccines. Prevention of viral gastroenteritis epidemics will rest with the identification of the vehicle of infection, interruption of the mode of transmission, and the potential development of vaccines.

INTRODUCTION

Gastroenteritis is one of the most common illnesses affecting infants, children, and adults and accounts for over 500,000 deaths annually in children under 5 years of age worldwide (1, 2). The term *gastroenteritis* implies an inflammation of the stomach and intestine, but, depending on the specific etiology, the pathophysiology of illness can be quite diverse. In fact, gastroenteritis can be caused by multiple different pathogens—viruses, bacteria, parasites—many of which produce no inflammation and some of which are increasingly being recognized as potential vaccine preventable diseases. The clinical presentation can vary widely from purely upper gastrointestinal symptoms of vomiting (e.g., winter vomiting disease) to acute diarrhea without any upper gastrointestinal complaints (3). Although gastroenteritis most often presents as mild diarrhea, it is a frequent cause of severe disease, leading to hospitalizations and deaths among infants, children, and the elderly, particularly among infants and children in developing countries. Acute gastroenteritis episodes are characterized by a range of symptoms, including abdominal cramping, malaise, anorexia, headache, myalgia, nausea, vomiting, and diarrhea. These symptoms can appear alone or together and can mimic illness caused by toxins, drugs, or other medical conditions. In this chapter, we will use the terms gastroenteritis and diarrhea interchangeably and will concentrate on those illnesses caused specifically by viruses.

HISTORICAL BACKGROUND

Our understanding of the role played by gastrointestinal viruses has been determined by our ability to detect these agents through direct observation, measurement of an immune response to infection or through genetic analysis of clinical specimens collected from patients (Table 1). Historically, viruses have been implicated as agents of acute gastroenteritis when no other pathogens could be identified in fecal specimens (4). Moreover, as recently as 1970, infectious agents could be identified in such a small percentage of patients with diarrhea that explanations such as the diarrhea of malnutrition, weaning, or physiologic constitution were invoked as the underlying cause of these disease episodes.

The ability to detect viral agents of gastroenteritis has followed the major historical advances in virology (Table 1). Early investigators demonstrated that "transmissible agents" present in fecal filtrates were able to transmit gastroenteritis to animals and humans (4). With the refinement of cell culture techniques for growing viruses from the 1950s through 1970s, a new generation of advances saw a number of viruses—echoviruses, adenoviruses, and coxsackie A and B viruses—isolated from fecal specimens of patients with diarrhea (5–8). Although these viruses were identified from patients with symptoms of gastroenteritis, establishing these agents as causes of disease has been challenging because these viruses have also been isolated from patients who had other syndromes or were asymptomatic.

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TABLE 1 Historical advances in the identification of viral agents of gastroenteritis

Year	Agent	Advance and comment
1940–1950	"Transmissable" agents	Fecal filtrates transmit gastroenteritis in animals and humans
1950–1970	Echoviruses, adenoviruses, Coxsackie A and B viruses	Viruses cultivated from stools of patients with diarrhea; causal relationship to disease unclear
1972	Norwalk virus	First virus clearly associated with diarrhea (9)
	Rotavirus group A	Virus discovered by Bishop et al. (10) in duodenal mucosa and now recognized as the most common cause of severe diarrhea in children
1975	Enteric adenovirus serotypes 40 and 41, group F	Unique serotypes and group of fastidious adenoviruses associated with diarrhea
	Astrovirus	Virus rarely identified in fecal specimens now recognized to be a more common cause of disease in children
1979	Calicivirus	"Classic" human calicivirus associated with disease in children, genetically related to the Norwalk family of viruses
1980s	Rotavirus groups B and C	Pathogens recognized in animals found to cause disease in humans; animal and human strains distinct
1970s–1980s	Norwalk-like viruses or SRSVs now recognized to be human caliciviruses, e.g., Snow Mountain agent, Hawaii agent, Toronto agent, minireovirus, Parramatta agent, Taunton agent, Montgomery County agent, and Desert Storm virus	Each virus morphologically identical but antigenically and genetically distinct; variant in the family *Caliciviridae*
1980s–1990s	Novel agents, e.g., torovirus, picobirnavirus, and enterovirus 22	Found in fecal specimens of patients with diarrhea more often than controls, but full association with disease is unclear
2000s–2015	Population based viral gastroenteritis burden studies	Following widespread rotavirus vaccine implementation, norovirus shown to be the most common cause of medically attended acute gastroenteritis in the U.S. (21)

In 1972, the Norwalk agent became the first virus discovered that was determined to be a causative agent of gastroenteritis (9). Using immune electron microscopy, Kapikian visualized grape-like clusters of small, round structured viruses (SRSVs) in fecal specimens of patients in an outbreak of diarrhea, but not in controls, and applied the same technique to document the patients' immune response. Since then, electron microscopy has been critical to identifying or confirming all the new viral agents of gastroenteritis including rotaviruses (10), adenovirus (11), astroviruses (12), and the "classic human" caliciviruses (13). Human caliciviruses have been placed in their own genus, Norovirus (previously called "Norwalk-like viruses"), along with Sapovirus (previously denoted as "Sapporo-like viruses") (14).

While many viruses have been identified in fecal specimens, the etiologic association of these viruses with disease requires further investigation and must meet four essential criteria for causality. In order to document an infection causing disease, the patient should exhibit a measurable immune response to the specific agent. The virus should also be present more often in patients with gastroenteritis than in persons without gastroenteritis (typically asymptomatic controls) (15). The onset of clinical signs and symptoms should temporally correspond with the onset of virus detection, and the termination of disease should in some way correspond with the end of detection. Some viruses from fecal filtrates have been given to animals to demonstrate the biological plausibility of illness, as well as to volunteers to fulfill Koch's postulates of disease causation. Consequently, while many viruses have been found in fecal specimens, some like torovirus (16), picobirnavirus (17), parechoviruses (15), coronaviruses (18), aichiviruses (19), and pestivirus (20), have yet to fulfill these strict criteria and be accepted as pathogens of the gastrointestinal tract in humans (15). These agents will require further laboratory, clinical, and epidemiologic investigations in order to confirm their association with gastrointestinal disease.

We now suspect that—at least in the United States—most gastrointestinal illnesses in children are due to viruses (15, 21). However, our understanding of the full spectrum of disease associated with these viruses, with the exceptions of rotavirus and to some extent norovirus, remains incomplete.

VIRAL AGENTS

The viral agents that are proven causes of gastroenteritis fall into four distinct families—rotaviruses (*Reoviridae*), human caliciviruses (*Caliciviridae*), enteric adenoviruses (*Adenoviridae*), and astroviruses (*Astroviridae*) (Table 2; Figure 1) (15). The diversity in viral genomic structures among these agents ranges from those containing single-stranded RNA (astroviruses, caliciviruses) to those with double-stranded RNA (rotaviruses), and to those with double-stranded DNA (adenoviruses). Despite the diversity of these agents and their epidemiologic characteristics, the clinical presentations of disease caused by these agents are indistinguishable. Moreover, while viruses in the same families also cause disease in animals, the amount of transmission between animals and humans is likely to be limited, if present at all. All of these viruses can be detected using electron microscopy, but the amount of virus shed in fecal specimens ranges from 10^{12} particles per gram (rotavirus) to subdetectable levels ($< 10^{6}$) in norovirus infections. Our knowledge of the epidemiology of these agents is a direct function of both this level of

TABLE 2 Microbiological and epidemiological characteristics of viral agents that cause gastroenteritis

Virus	Microbiological characteristics		Epidemiological characteristics	
	Morphology	Detection method	Setting	Comment
Rotavirus				
Group A	70 nm double-capsid wheel-shaped ("rota") virus with 11 segments of dsRNA	EIA, EM, PAGE, RT-PCR, culture	Endemic in children	Globally, the most common cause of severe diarrhea in children; accounts for ca. 13% hospitalizations for diarrhea in U.S. children; mode of transmission unknown
			Outbreaks in high-risk groups in adults	Affects travelers, immunocompromised patients, parents of infected children, elderly residents of aged-care facilities, and caretakers in day care centers
Group B	Same as group A	EM, PAGE, EIA, RT-PCR	Epidemic	Associated with cholera-like disease in adults; transmitted by water
Group C	Same as group A	EM, PAGE, EIA, RT-PCR	Epidemic	Worldwide distribution; outbreaks in children (newborns to school-age); mode of transmission and prevalence of outbreaks unknown
Noroviruses (human calicivirus)	27 to 32 nm SRSV with ssRNA	EM (IEM), RT-PCR/ Southern blot hybridization; RT-PCR; seroconversion using expressed antigens	Epidemic and endemic in all age groups	The most common cause of childhood diarrhea in the U.S.; accounts for 17% of diarrhea hospitalizations in children aged <5; most common cause of sporadic gastroenteritis and diarrheal outbreaks; all age groups affected; transmission via person to person contact, contaminated food, shellfish, and water
Sapoviruses (human calicivirus)	Distinguishable by EM from Norwalk by calices and Star-of-David morphology; otherwise identical	Expressed antigens not available; otherwise identical	Endemic in children	Mode of transmission unknown
Adenovirus, enteric (serotypes 40,41)	70 nm icosahedral virus with dsDNA; serotypes 31, 40, and 41 associated with diarrhea	EM, EIA for hexon and serotypes 40 and 41	Endemic in children	Disease may be more severe than rotavirus-induced diarrhea
Astrovirus	27 to 32 nm, ssRNA small round virus (star configuration)	EM, EIA, RT-PCR	Endemic in children; may cause outbreaks	All children infected in first 3 years of life; less severe than rotavirus; mode of transmission unknown; outbreaks reported in day care centers, schools, and nursing homes; winter seasonality

EIA, enzyme immunoassay; EM, electron microscopy; PAGE, polyacrylamide gel electrophoresis; IEM, immunoelectron microscopy; dsRNA, double-stranded RNA; ssRNA, single-stranded RNA.

shedding and the quality of techniques available to detect the virus present in fecal specimens.

EPIDEMIOLOGY

Viral gastroenteritis occurs in two distinct epidemiologic settings: childhood diarrhea (i.e., endemic disease) and outbreaks (i.e., epidemic disease) (Table 3). The majority of the diarrheal illnesses in children aged less than 5 years is due to a variety of viral agents—rotavirus (15, 21–23), adenovirus (11, 15, 24–27), calicivirus (15, 21, 28), and astrovirus (15, 27, 29). Infants may be infected in the first few months of life, and the prevalence of antibody to these agents approaches 100% by 5 years of age (30).

Globally, rotavirus is the most common cause of severe gastroenteritis in children <5 years of age, accounting for approximately 200,000 deaths per year in children less than 5 years of age, with deaths among children in the poorest

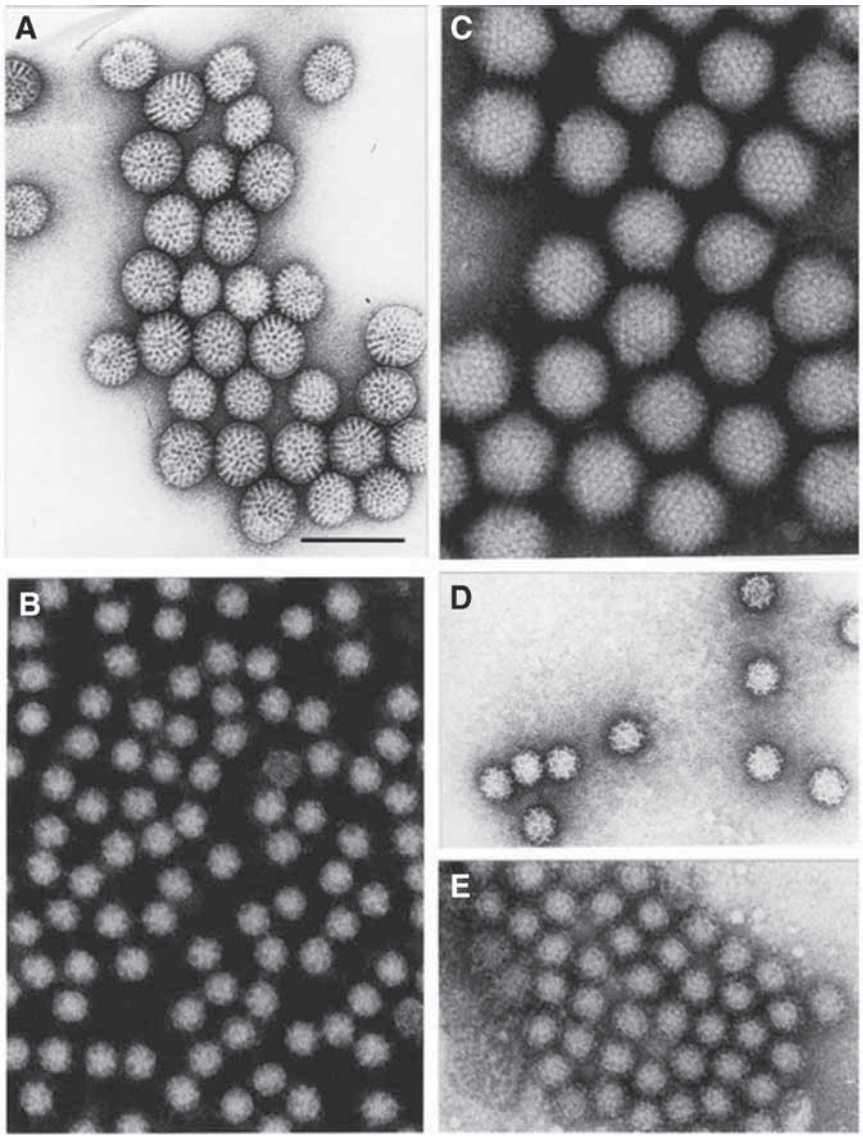

FIGURE 1 Electron micrographs showing agents of viral gastroenteritis. (A) rotavirus; (B) astrovirus; (C) adenovirus; (D and E) Norwalk-like viruses of the family *Caliciviridae*.

countries accounting for more than 85% of the total (1, 31); however, widespread implementation of highly effective rotavirus vaccines since 2006 has resulted in a dramatic shift in the epidemiology of pediatric viral gastroenteritis in early introducer countries. Substantial reductions in the burden of severe rotavirus gastroenteritis were observed following rotavirus vaccine implementation (21, 32–34), and in many countries, noroviruses have overtaken rotavirus to become the predominant cause of severe gastroenteritis in the pediatric population (21, 35, 36).

TABLE 3 Contrasting epidemiological patterns of viral gastroenteritis

Characteristic	Pattern in:	
	Childhood diarrhea (endemic)	Outbreaks (epidemic)
Viruses	Rotavirus group A, human caliciviruses, adenovirus, astrovirus	Human caliciviruses, rotavirus groups B and C ± astrovirus or rotavirus (special settings)
Age	< 5 yr	All ages
Antibody interpretation	Seroprevalence is 100% by age 5	Seroprevalence is variable but seroconversions in affected cases
Mode(s) of transmission	fecal-oral, contact, droplet	Person-to-person, food (shellfish), water
Prevention and control	Effective vaccines for rotavirus licensed and in use	Public health measures to stop transmission by disinfection, exclusion of ill persons, and removing contaminated food or water

Norovirus is estimated to cause 18% of severe diarrheal disease in children under the age of 5 years worldwide; 17% of inpatient cases and 24% of community episodes (37). Following implementation of rotavirus vaccination, among children under 5 years old in the United States studied in an active surveillance network, norovirus accounted for 21% of gastroenteritis requiring medical attention—17% among inpatient cases and 28% of community episodes (21). Other important viral causes of gastroenteritis include adenoviruses, and astroviruses accounting each for about 5 to 10% of acute gastroenteritis episodes in medically attended children. Recently, adenoviruses 40/41 were found in 12% of children aged < 5 years suffering from acute gastroenteritis in the United States, and astroviruses were detected in 5% of children in this cohort (15).

All the major enteric viruses are transmitted primarily through close person-to-person contact via the fecal-oral route (38). Noroviruses are present in vomitus of ill people. Droplet spread through exposure to vomitus has been demonstrated to be a mechanism of transmission both in healthcare and community settings (39, 40). Additionally, noroviruses are spread through contaminated food and therefore are a major cause of foodborne and occasionally waterborne disease (41–43). The modes of transmission of adenovirus are less well understood, but it is presumed to be primarily through close contact by fecal-oral spread. Spread through fomites is possible for each of the agents, and may play an important role in disease acquired in institutional settings and group childcare (44). Epidemiologic studies suggest that transmission within families, communities, and special settings may occur despite the availability of sanitary food and water.

Outbreaks of viral gastroenteritis occur in all age groups and in many different settings (43, 45). Since viral gastroenteritis is often mild, we know much more about large outbreaks that occur in identifiable settings (e.g., weddings, cruise-ships, long-term care facilities, hospital wards), than small outbreaks or sporadic cases, where the source of infection cannot easily be traced (3). Noroviruses are the leading cause of reported outbreaks of acute gastroenteritis in the United States (42, 46). Outbreaks of norovirus infections are also frequently reported in institutional settings, such as nursing homes and day care centers (45). Large outbreaks of group B rotavirus have also been well documented in China (47), and smaller outbreaks of group C rotavirus have been identified among children and adults in a global distribution (48, 49). Outbreaks of astrovirus (50) and rotavirus (51, 52), two endemic viruses of children, have also been documented among people who should already be immune following their first infections as children. Rotavirus outbreaks among attendants in day care centers (53), mothers of children with rotavirus (53), travelers (54), formal dinner attendants (55), retirement communities (52), and patients in long-term care facilities (56), may be due to alternate modes of transmission in which direct contact with a large fecal inoculum overwhelms an individual's preexisting immunity.

In epidemics of viral gastroenteritis, particularly those caused by noroviruses, a mode of transmission can often be documented (46, 57). Contaminated food (43, 58), especially raw shellfish (59) and water (60), are commonly identified vehicles for transmission, perhaps because the spread of viruses by airborne droplets (61), vomitus (62), or direct person-to-person contact (57, 61) is more difficult to prove (45). Even produce, such as raspberries, contaminated before retail distribution, may be a source of infection (63). Contamination of environmental surfaces with noroviruses has occurred during outbreaks in institutional settings and may serve as a reservoir that sustains an outbreak (64). Inapparent contamination of restrooms may be a source of infection in many settings (65). Primary prevention efforts can be directed at interrupting transmission by removing the contaminated vehicle of infection.

While rotaviruses and noroviruses occur in distinct epidemiologic patterns, there is considerable crossover in presentation. Winter seasonal epidemics among infants and children are the most common presentation of rotavirus, but these agents do cause disease in adults and the elderly, suggesting that these groups may be exposed to unusually large inocula in the context of special settings, or that immunity to rotaviruses wanes over time (52). Epidemics of caliciviruses are common in older children and adults despite the fact that most children possess antibody to both genuses of the family *Caliciviridae*—noroviruses and the sapoviruses (66). Noroviruses are now known to be common in children presenting to medical care (15, 21). Although a rapid and accurate diagnostic assay is not commonly available for diagnosing norovirus infections, epidemiologic features are useful in confirming norovirus as a cause of outbreaks (67). Epidemiologic criteria were validated to be highly specific in discriminating norovirus outbreaks from other etiologies and could continue to be used until diagnostic assays become widely available (68).

Risk factors for fatal disease include social- and healthcare system characteristics such as the access to proper rehydration therapy, and biological factors such as the nutritional status and immunocompetence of the child (33, 69). Diarrheal deaths are not uncommon in the elderly (70, 71), and have been identified among patients who became ill in norovirus outbreaks (72). In these patients, electrolyte disturbances and secondary infections in patients with preexisting health problems have appeared to place these individuals at particular risk (72).

CLINICAL MANIFESTATIONS AND PATHOGENESIS

Viral infections of the intestinal tract cause clinical syndromes that can range from asymptomatic infections to severe, complicated, dehydrating diarrhea, and death. In infants and young children, mortality rates due to rotavirus are particularly high in developing low-income countries (1, 31). The key clinical feature determining disease severity of viral gastroenteritis is the degree and rate at which fluids and electrolytes are lost, and the rapidity with which these losses can be replaced by oral or parenteral rehydration therapy (73).

Despite the variety of viral agents that cause gastroenteritis, a number of key features in their epidemiology and clinical presentation can be explained by the pathophysiology of infection and disease. For those agents studied, the inoculum size is small; fewer than 100 norovirus viral particles properly buffered can cause disease (74). The small inoculum size would permit transmission by airborne droplet spread or direct contact, although the importance of these modes of spread has been difficult to document (57, 61, 62). Following ingestion but prior to the onset of clinical manifestations, the virus replicates in the epithelial cells of the small intestine during an incubation process that can range from 12 to 48 hours, depending upon the inoculum size (75). The incubation period of 12 to 36 hours for viral gastroenteritis (e.g., norovirus) helps in distinguishing these agents

from organisms that produce a preformed bacterial toxin (e.g., *Staphylococcus aureus* or *Bacillus cereus)* and typically have a shorter incubation period (<12 hours).

Viral replication may be associated with a low-grade fever, myalgia, and malaise. Disease is generally of short duration and lasts from 3 to 5 days, perhaps representing the time period required for the small intestine to replace cells damaged by infection and to mount the immune response necessary to clear the infection (76). Shedding of virus can persist at low levels for days to weeks after the clinical illness, depending upon the sensitivity of the assays used for detection (44).

Acute episodes of viral gastroenteritis are distinguished by the presence of watery diarrhea and vomiting. A majority of patients with diarrhea experience vomiting as an associated symptom, but some patients may present with vomiting alone (e.g., winter vomiting disease) (77). The mechanism of emesis during viral gastroenteritis is poorly understood, but is likely different from the mechanism of the associated secretory diarrhea. While diarrhea was traditionally believed to result from cellular damage in the intestine, some data for rotavirus indicate that tissue invasion may not be necessary to cause disease (78), as inactivated rotavirus can cause a secretory diarrhea in animal models. Although bloody diarrhea has been occasionally described in the context of rotavirus infections, classic dysentery associated with tissue invasion and an intense cellular infiltration of the intestinal mucosa is not recognized in viral gastroenteritis. Indeed, the lack of blood in the stool distinguishes viral diarrheas from the bacterial or amoebic dysenteries. While viral gastrointestinal infections are generally confined to the intestine, rotavirus and norovirus infections can result in antigenemia and the presence of nucleic acid in the blood of ill patients (79). While extraintestinal disease is rare, asymptomatic infection is common, especially for norovirus (28), and is thought to play a role in disease transmission (80).

Chronic, prolonged diarrhea may be associated with coronaviruses and picobirnaviruses, specifically in patients infected with HIV (81); however, proof of pathogenicity has not been established (82).

HIGH-RISK GROUPS

Hospital wards, day care centers, and extended care facilities for the elderly provide special settings where outbreaks of viral gastroenteritis commonly occur (45, 52, 83). Similarly, infections with viral agents are common in persons traveling from well-resourced countries to middle and low-income countries (84). Even childhood pathogens such as rotavirus can cause gastroenteritis in immune travelers, suggesting that the inoculum to which they are exposed may be large enough to overwhelm preexisting immunity and involve alternate routes of transmission, such as the respiratory tract (54). Viral gastroenteritis remains an important cause of illness among U.S. military personnel, accounting for an estimated 68% of all acute gastroenteritis episodes during 2002 to 2012 (85). Large outbreaks of gastroenteritis due to noroviruses have also been documented among tourists aboard cruise ships (65) and on commercial airplane flights (86), where viral gastroenteritis can spread rapidly, disable passengers and crew, and pose challenging problems for control (62).

Some populations are at particularly high risk of viral gastroenteritis due to either their increased exposure to the viruses or their increased susceptibility to infection (Table 4).

TABLE 4 Groups at high risk for viral gastroenteritis

Increased exposure to viruses
Children and the elderly
Parents and caretakers of children
Hospital wards, day care centers, nursing homes
Travelers in developing countries
Increased susceptibility
Children and the elderly
Immunodeficient
Congenital, e.g., SCID
Acquired, e.g., HIV infection
Chemotherapy, e.g., bone marrow transplant
Individuals with functional FUT2 enzyme (i.e., secretors)

The predisposition of young children and the elderly to viral gastroenteritis probably reflects a lack of immunity, or waning immunity with age, plus a concentrated exposure to the agents in settings such as day care centers or long-term care facilities, where hygienic precautions can be easily breached (52, 87, 88).

In the early studies with volunteers challenged with noroviruses, approximately 13 to 40% of volunteers never became infected, and only 50% developed illness. Further investigations showed that susceptibility to norovirus infection, and possibly rotavirus infection, depends on the presence of histocompatibility-blood group antigen (HBGAs) receptors in the guts of susceptible hosts (89–93). HBGAs are carbohydrates expressed on mucosal epithelia, which are recognized as receptors allowing norovirus attachment and cellular entry (89). The expression of HBGAs is determined by three gene families expressing the ABO (A/B enzymes), secretor (FUT2) gene, and Lewis-type (FUT3). Single nucleotide gene polymorphisms can inactivate the expression of these gene products, interrupting norovirus binding and the infection process. Mutations in the FUT2 gene leading to the absence of HBGA expression (nonsecretor phenotype) have been associated with resistance to norovirus infection, in particular to the GII.4 genotype, which is the predominant genotype worldwide (89, 90, 94–96). The host-specificity may also explain why persons with higher levels of preexisting antibody to noroviruses were more likely to develop illness on rechallenge with norovirus (97). Those without antibodies to noroviruses may not get infected because they lack genetic susceptibility to infection and thus never mount an immune response to that particular strain. Recent evidence points to lower susceptibility of nonsecretors to rotavirus infections in various populations (91–93).

Children, as well as adults, with congenital or acquired immunodeficiencies are easily infected with viral agents that cause gastroenteritis and often experience prolonged shedding of these viruses (82, 98). Severe and prolonged diarrhea associated with each agent has been reported among children with malnutrition, and among children with congenital or acquired immunodeficiencies (82, 99–102). In HIV-infected patients, in whom viruses have been intensively sought, both common viruses (rotaviruses, enteric adenoviruses), as well as the less common agents (astroviruses and caliciviruses) have been identified (101); however, in a large study in Malawi, severity and duration of clinical symptoms from rotavirus disease were no different in children with and without HIV infection (103).

OTHER VIRAL AGENTS OF GASTROENTERITIS

In addition to detection of picobirnaviruses in immunocompromised patients, other viruses have been identified in humans in association with gastroenteritis. Toroviruses (enveloped, positive-stranded RNA viruses in the family *Coronaviridae*) have been shown to cause infection and diarrhea in animals, but their role as a cause of gastroenteritis in humans remains unclear (104, 105). Toroviruses have been identified by electron microscopy in human stool with confirmation by enzyme immunoassay (EIA) using reagents to bovine toroviruses. A survey of specimens found torovirus present in 8% of 2,800 specimens screened (105). One study reported torovirus detection among 35% of 206 hospitalized children with nosocomial gastroenteritis, compared with 14% of 206 controls without gastroenteritis (104). Patients infected with torovirus were more often immunocompromised and infected in the hospital compared with patients infected with rotavirus or astrovirus. Clinically, patients with torovirus exhibit bloody diarrhea more often, but vomiting less often compared with patients infected with rotavirus or astrovirus.

Coronaviruses are important causes of respiratory infections in humans and other species, and have been identified as a cause of gastroenteritis in several animal species. Earlier studies in humans found coronavirus in association with diarrhea and tropical sprue (18, 106). Coronaviruses have been observed by electron microscopy in 0 to 6% of stool specimens examined (107, 108). In 2002, a novel coronavirus was identified as the cause of the newly emerging severe acute respiratory syndrome (SARS). Interestingly, over a third of the SARS patients had diarrhea and in some, diarrhea was the presenting symptom (109). Fecal-oral transmission was documented and fecal shedding occurred in one patient for 73 days after onset of illness (110). Gastrointestinal coronavirus infection during global SARS outbreak demonstrates the public health and clinical importance of this finding with regard to interruption of fecal-oral transmission and use of stool samples to diagnose illness, since stool samples have the highest yield for SARS and pose less risk for transmitting illness to the healthcare workers collecting the samples. The recently described Middle East Respiratory Syndrome coronavirus (MERS-CoV) has been associated with diarrhea in a large proportion of patients (111), and MERS-CoV RNA has been detected in blood and stool in some patients. In the future, use of molecular-based diagnostic tests may help clarify the role of coronaviruses as a cause of both sporadic and epidemic gastroenteritis in humans.

It should be noted that gastroenteritis has been increasingly recognized with other respiratory infections, particularly avian and pandemic influenza, and, more recently, Ebola.

ASSOCIATED DISEASES

The group of viruses that cause gastroenteritis in humans also cause a variety of other illnesses in other animal species. Rotavirus and astrovirus can cause fatal hepatitis in SCID mice and normal ducks, respectively, and a calicivirus (hepatitis E virus) causes hepatitis, while other caliciviruses cause a variety of bullous lesions in many animal species (112, 113). Nonenteric adenoviruses have a wide diversity of clinical presentations in humans. Yet, despite these biological similarities, it has been difficult to document important extraintestinal manifestations of this group of human gastrointestinal viruses in humans.

Rotavirus has been found in extraintestinal sites such as the hepatic tissue of children who have died with SCID (114) and in the cerebrospinal fluids of children with rotavirus diarrhea who had convulsions (115, 116). These systemic complications have been unusual findings, but antigenemia and viremia after rotavirus infection may be more common than previously suspected (115). A reduction in childhood seizures has been associated with rotavirus vaccination of children (117). While rotavirus detection has been reported in children with a wide variety of clinical problems—including gastrointestinal bleeding (118), Sudden Infant Death Syndrome (119), ulcerative colitis (120), Reyes syndrome (121), Kawasaki disease (122), intussusception (123), and necrotizing enterocolitis (124)—the association with disease has not been confirmed and rotavirus is probably not the causal agent of disease for most of these conditions.

DETECTION METHODS

The greatest impediment to our understanding of the viral agents of gastroenteritis has been the lack of simple and sensitive diagnostic tests—with the exception of tests for rotavirus—that would permit physicians to make a rapid diagnosis and epidemiologists to study the burden and spread of disease. A diagnosis of viral gastroenteritis rests on finding the virus or one of its components in a fecal specimen, or detecting a rise in significant antibody titer in the sera of an infected patient. Shortly after the first identification of rotavirus as a cause of infant gastroenteritis, simple enzyme immunoassays were developed that were sensitive, specific, inexpensive, and easy to use in the field (125, 126). Currently, commercial diagnostic tests based on immunoassays are available to detect rotavirus, norovirus (low sensitivity), and adenovirus in fecal specimens.

Molecular diagnostic methods have markedly improved understanding of the etiology of gastroenteritis, e.g., documenting that noroviruses are one of the leading causes of diarrhea worldwide (22, 127, 128). Real-time (quantitative) PCR (RT-PCR) is the preferred laboratory method for detecting noroviruses. These assays are highly sensitive and are able to detect as few as 10 norovirus copies per reaction and provide a semiquantitative estimate of viral load. The assay is generally used to detect norovirus in stool, but can also be used for vomitus, foods, water, and environmental specimens for outbreak investigations, though with reduced sensitivity. Norovirus genotyping is conducted by sequence analysis of the RT-PCR products (129). Given the exquisite sensitivity of RT-PCR for norovirus, and the high frequency with which the virus can be found in healthy individuals (21, 28), diagnostic results should be interpreted in the light of clinical characteristics and, if available, the background level of detection in a control population. A commercial EIA for norovirus was approved in the United States in 2011; however, these assays have low sensitivity and are not recommended for diagnosing norovirus infection in sporadic cases (130–132). Norovirus EIAs may be useful in outbreak investigations, where even confirmation of norovirus in a proportion of patients would help determine the etiology of the outbreak.

Caliciviruses and other viruses can sometimes be visualized by electron microscopy in fecal specimens if the concentration of virus exceeds 10^{6-7}/gram of stool, the threshold for detection (133). It is not surprising that we know most about rotavirus since this virus is shed in huge numbers (up to 10^{12}/gram of stool) during the acute illness. Unfortunately some viruses (e.g., astrovirus, calicivirus) often cannot be

visualized by electron microscopy even during acute diarrheal episodes, indicating that their concentration in stool is below the level of detection. Virus is shed in greatest concentrations during the period of acute diarrhea. Thus, fecal samples collected within 48 hours of disease onset are most likely to yield a positive diagnosis. Virus can be shed in smaller quantities for several hours before the onset of disease and for days to weeks after the illness is resolved, but special research techniques (e.g., nucleic acid detection, cultivation) are needed for detection (134).

For epidemiologic research, serosurveys can provide additional understanding of the extent of infection. While current methods may not detect virus in fecal specimens, documentation of a rise in antibody titer to a specific agent can help confirm that the patient was infected, even though the patient's illness has usually passed. For norovirus, serology has long been utilized for outbreak investigations, since it is often easier to collect and test paired sera from many patients than it is to detect virus in stool (135). Seroprevalence studies have also increased appreciation of caliciviruses as a common infection in young children, and the virus can now be detected in their stool samples (66, 127). The discrepancy between a high seroprevalence of antibodies to the virus, but low rates of detection noted in the past, can now be explained because past assay of virus in stool specimens were quite insensitive (136). Serologic assays have been developed for the other viral agents of gastroenteritis (e.g., groups B and C rotavirus, astrovirus), but are only available in reference laboratories.

Efforts to cultivate noroviruses in available cell culture systems or to develop an animal model have long been unsuccessful (137). Recently, B cells were identified as a cellular target of human noroviruses and enteric bacteria as a stimulatory factor for norovirus infection, leading to the development of an *in vitro* infection model for human noroviruses but this system is not widely available (138).

TREATMENT AND PREVENTION

No virus-specific therapies are available for viral gastroenteritis. Case management depends on accurate and rapid assessment of the severity of dehydration, correction of fluid loss and electrolyte disturbances, and maintenance of adequate hydration and nutrition (73). In 2004, the WHO and UNICEF recommended low-osmolarity Oral Rehydration Solutions (ORS) (245 milliosmoles/liter), which were shown to exert decreased stool output and vomiting in comparison with children treated with traditionally recommended ORS (311 milliosmoles/liter).

Oral rehydration therapy may also be used for traveler's diarrhea of either viral or bacterial origin (139). Breastfed infants should continue to nurse on demand. As tolerated, patients should begin taking food early in the illness since adequate caloric intake has been found to enhance patient recovery.

Intravenous rehydration may be required for children with severe dehydration ($\geq$10% fluid deficit, shock, or near shock), intractable vomiting, or ORS failure. Factors such as young age, unusual irritability or drowsiness, progressive course of symptoms, or uncertainty of diagnosis might indicate a need for close observation (73).

The volume of fluid to be replaced may be assessed clinically by determining the severity of dehydration. When severe dehydration is present, more rapid fluid replacement may be necessary using intravenous fluids (140). Great attention must be paid to infants and younger children presenting with diarrhea, vomiting, and clinically significant dehydration (i.e., moderate-severe dehydration). Such patients require early oral or parenteral fluid plus electrolyte replacement. In the case of rotavirus diarrhea, vomiting and diarrhea may occur together, but diarrhea and subsequent dehydration often persist from 4 to 8 days after onset, necessitating persistent and regular fluid replacement (140). In some settings, elderly patients may develop acute vomiting and diarrhea. Such illnesses in high-risk elderly patients who may be immunocompromised or undernourished, or who have underlying diabetes or heart disease, must be recognized as potentially life-threatening (141). Given these risk factors for gastroenteritis in the elderly, prompt attention and treatment of dehydration and electrolyte imbalances during acute gastroenteritis may be critical to patient survival. Routine infection control procedures during care of ill residents, including hand-washing and barrier precautions (e.g., gloves), should help reduce transmission of viral gastroenteritis in long-term care facilities for the elderly.

There is no role for antibiotic therapy for the treatment of uncomplicated viral gastroenteritis in children. Indiscriminant use of antibiotics may result in adverse consequences such as spread of antibiotic resistant bacteria and treatment–related adverse events. While diphenoxylate or loperamide may reduce symptoms such as abdominal cramping or stool frequency, they have not been demonstrated to reduce intestinal fluid losses, have no practical value, and should be avoided since they may be harmful in some cases, particularly those younger than 3 years of age (142, 143). There is conflicting evidence regarding the use of oral probiotics, such as *Lactobacillus* species, to reduce the duration of diarrhea caused by rotavirus (144–146). Zinc, used both as supplement and treatment, reduces severity, duration, and incidence of diarrhea in low-middle income countries and is considered one of the mainstays of pediatric acute gastroenteritis treatment in developing countries (147, 148).

Those patients in the special populations discussed above with persistent or chronic diarrhea may need additional nutritional support in conjunction with fluid and electrolyte replacement. Selected children who are immunodeficient and develop chronic rotavirus illness may be treated with oral feedings of human milk containing antirotavirus antibody (149). At the present time, no specific antiviral agents have been recommended to treat gastroenteritis due to viral agents, although several agents including protease inhibitors have been studied (150).

While no chemoprophylaxis for agents of viral gastroenteritis is currently available, many investigators feel that breast milk protects against the development of clinically severe rotavirus diarrhea with dehydration in feeding infants (151, 152). In premature infants, oral human serum globulin that contains rotavirus antibody has been administered prophylactically and shown to protect against rotavirus gastroenteritis. Also, bovine colostrum containing antirotavirus antibodies has been administered to infants and young children as a form of passive immunization, and this was found to prevent rotavirus diarrhea (152).

Prevention of viral gastroenteritis depends upon the epidemiologic setting. For childhood disease, prevention strategies other than vaccination may be of limited value. Recommendations include proper diaper handling and disposal of feces by caregivers, double-diapering of infants, routine hand-washing, and use of barrier precautions to reduce transmission in hospital and day care settings. For outbreaks associated with norovirus, identification of the

mode of transmission can lead to specific public health interventions to remove contaminated foods or water.

In 2006, two live oral rotavirus vaccines—pentavalent human-bovine reassortant RotaTeq (Merck Vaccines, Whitehouse Station, NJ) and monovalent attenuated human Rotarix (GlaxoSmithKline Biologicals, Rixensart, Belgium)—were licensed for use in many countries. Large prelicensure clinical trials of each of these vaccines have demonstrated high efficacy (85 to 98%) against severe Group A rotavirus (i.e., the most common cause of endemic diarrhea in children < 5 years of age) disease and a good safety profile (153, 154). The use of these vaccines was shown to be highly effective in reducing rotavirus disease burden and the overall impact from diarrhea among children in the several countries, including high and middle income settings (33, 34, 155, 156). A third live attenuated oral monovalent human-bovine (116E) rotavirus vaccine has recently been successfully tested in Indian infants (157).

The observation that adults are at risk of repeated infections with the caliciviruses suggests that either immunity is short-lived (158), or that the antigenic diversity of strains is too great to permit natural immunity to all the virus strains most often responsible for human disease (159); however, several human norovirus vaccine candidates are in various stages of clinical trials (160, 161). In a norovirus challenge trial of a vaccine candidate composed of bivalent virus-like particles (VLPs), the vaccine reduced vomiting and diarrhea in healthy volunteers (161). Phase 3 trials are about to begin for this vaccine candidate. Vaccines against other viruses may be warranted when the full disease burden of these infections can be fully assessed.

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Viral Hepatitis

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5

Viral hepatitis describes a characteristic clinical syndrome resulting from necro-inflammatory pathology of the liver that is caused by one of the recognized hepatitis viruses. There are five established human hepatotropic hepatitis viruses: hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis D virus (HDV), and hepatitis E virus (HEV). Acute hepatitis can also be a manifestation of other systemic viral infections, including herpesviruses (Epstein-Barr virus, cytomegalovirus, and herpes simplex virus) and yellow fever. Although the human hepatitis viruses can all cause the syndrome of acute hepatitis, they have distinct virology, phylogeny, routes of transmission, and risk of chronicity. In this chapter, we discuss the typical clinical syndromes and pathological features of viral hepatitis due to the hepatitis viruses A-E (Table 1), as well as the diagnostic evaluation of patients presenting with suspected viral hepatitis.

Historical Perspectives

The identification and characterization of the different hepatitis viruses has been among the most significant medical breakthroughs in the last 50 years, leading to effective vaccines and drug development that will save millions of lives. The first account of the clinical syndrome of hepatitis dates as far back as the 3rd millennium BC, based on descriptions of jaundice engraved on Sumerian clay tablets. In 460 BC Hippocrates described epidemic jaundice and fulminant liver failure in patients who died within 11 days (1). By the Middle Ages, jaundice had become a well-recognized entity, although its etiology remained anchored in mythical roots. The first description of an epidemic of "serum hepatitis" was documented by Lurman in 1885 among 191 German ship workers in Bremen after a smallpox vaccination campaign using human lymph (2). Multiple outbreaks of acute hepatitis have been reported during military campaigns. The largest documented outbreak of "serum hepatitis" occurred in 1942 when 50,000 U.S. army men were hospitalized with jaundice following vaccination with yellow fever vaccine that was contaminated by plasma, in an epidemic affecting 300,000 servicemen. (3, 4). This outbreak led to the conclusion that there was an infectious agent in the human lymph administered with the smallpox vaccine, a conclusion subsequently confirmed by the prospective demonstration that viral hepatitis was transmissible to volunteers following administration of filtered inocula (5).

The existence of more than one hepatitis virus was first proposed in 1946 by MacCallum with the suggestion of "serum" vs "infectious" hepatitis (6), now recognized as associated with HBV and HAV, respectively. A series of experiments from 1958 to 1964 led by Krugman and colleagues at the Willowbrook State School in Staten Island, NY, confirmed the distinct incubation periods and primary modes of transmission of serum and infectious hepatitis (7). These experiments also demonstrated the protective effects of inoculating susceptible individuals with boiled HBV serum as well, the first step towards developing an effective HBV vaccine (8, 9). This work was not without controversy, involving the intentional infection of mentally disabled children and, with the Tuskegee Syphilis Study (10), was one of a number of clinical experiments leading to the National Research Act (1974) and the Belmont Report: Ethical Principles and Guidelines for the Protection of Human Subjects of Research (1979) in the United States, the foundation for guidelines for ethical human research. Despite the recognition of the distinct clinical syndromes of "serum" and "infectious" hepatitis, the causative agents remained elusive, until 1964 when Blumberg and colleagues serendipitously discovered a novel antigen in the serum of an Australian Aborigine that precipitated antibody in patients with acute leukemia who had received multiple blood transfusions. Termed the *Australia antigen*, it was initially postulated as a biomarker to aid the diagnosis of leukemia. Three years later, the association between Australia antigen and "serum" (posttransfusion) hepatitis was recognized (11). The Australia antigen, the HBV surface antigen (HBsAg) that forms the viral envelope, would become the prototypal seromarker of HBV infection (12). Blumberg would go on to win the Nobel Prize in medicine for his work. Dane and colleagues later took HBsAg positive sera and visualized the complete hepatitis B virion using electron microscopy (13). HBV was found to be a 3020–3320 nucleotide DNA virus of the family *Hepadnaviridae* (Table 1). More detailed virological characterization followed, with the identification of the HBV e protein (HBeAg) (14), and the later definition of HBeAg-negative chronic hepatitis B (15, 16). HBsAg and HBeAg were identified as biomarkers for risk of hepatocellular carcinoma (HCC) (17), with prospective studies then showing that much of this risk was explained by persistent

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TABLE 1 Clinical and epidemiological features of viral hepatitis

Feature	HAV	HBV	HCV	HDV	HEV
Classification	*Picornavirus* ssRNA (+)	*Orthohepadnavirus* dsDNA	*Flavivirus* ssRNA (+)	*Deltavirus* ssRNA (-)	*Hepevirus* ssRNA (+)
Incubation period (clinical)	15–45 d	45–160 d - HBV DNA detectable 10–30 d - HBsAg detectable 3 weeks after HBV DNA	10–160 d - HCV RNA detectable 10–50 d - Anti-HCV detectable 4–10 wks	30–60 d	15–60 d
Development of chronic hepatitis	0	Neonates >90% Adults <5%	50–75%	Common (invariable in superinfection)	<1% (chronicity reported in immunosuppressed)
Transmission					
- Enteric	+++	-	-	-	+++
- Percutaneous	-	+++	+++	+++	-
- Sexual	-	++	+	++	-
- Perinatal	-	+++	+	+	-
Acute liver failure	0.1%	0.1–1%	Rare	5–20%	1–2% (10–20% in pregnancy)
Prognosis	Excellent	Liver related mortality associated with chronic infection = 15–40% without treatment	Variable Liver related morbidity more common in patients with comorbidities including alcoholism and nonalcoholic steatohepatitis	Chornic infection has a poor prognosis	Good except in pregnant women
Vaccine	10 year protection	3 injections, lifetime protection	None available	None available	Investigational (approved in China)

high-level HBV DNA replication (18, 19), a key determinant of long-term risk of liver cirrhosis and HCC. The development of experimental models of HBV replication, and recognition of the similarity between the HBV DNA polymerase and the HIV reverse transcriptase, has also led to effective nucleos(t)ide analogue therapy for HBV, starting with lamivudine in 1998 (20), paving the way for the current first-line agents entecavir and tenofovir.

An effective HBV vaccine has been available since 1982 (21). The first plasma-derived HBV vaccine was developed following the recognition by Krugman and colleagues that boiling serum containing HBV inactivated the virus but preserved HBsAg antigenicity. Soon after, the second generation vaccine containing recombinant HBsAg was released. The HBV vaccine is on the World Health Organization (WHO)'s list of essential medicines. WHO recommends universal neonatal HBV vaccination. In countries with low or intermediate endemicity, all children and adolescents younger than 18 years old and not previously vaccinated should receive the vaccine. Adults in high-risk groups in these countries should also be vaccinated, including people who inject drugs, people in custodial settings, men who have sex with men, family members of people with hepatitis B infection, and healthcare workers. In high prevalence countries, HBV vaccination programs have now been shown to reduce the prevalence of chronic hepatitis B infection, as well as rates of fulminant hepatitis and hepatocellular carcinoma in young adults. It is possible with broad vaccine coverage that HBV could be eliminated as a public health problem.

The first description of "infectious" hepatitis, or HAV, was in the early 20th century in separate reports by Cockayne and Blumer (22, 23). They described epidemic outbreaks of hepatitis that were not related to parenteral exposures. These outbreaks were transmitted by person to person contact, developing 7 to 10 days following exposure, and mostly affected children and young adults. Krugman later confirmed the distinct mode of transmission and incubation period of "infectious" hepatitis in his Willowbrook hepatitis studies, but the virus itself remained elusive. Hepatitis A virus was finally identified in 1973 by Feinstone and colleagues using electron microscopy to examine filtrates of feces from infected individuals (24). Detailed virological, serological, and clinical characterization showed that HAV was an RNA virus of the family *Picornaviridae* causing acute, transient hepatitis (Table 1). The virus was grown in tissue cultures and an inactivated HAV vaccine was developed 20 years later. Despite vaccination programs, more than 1.5 million cases of symptomatic HAV infection continue to occur annually. Levels of endemicity correlate with hygienic and sanitary conditions, with most cases reported in the developing world.

Hepatitis D virus, also known as delta virus, was identified next, when in 1977 Rizzetto and colleagues first

detected a novel "delta" antigen in the nucleus of hepatocytes in HBsAg-positive patients (25). Clinical and chimpanzee studies then demonstrated the dependency of the HDV life cycle on coinfection with HBV. HDV is a defective RNA virus that requires the presence of HBsAg for viral packaging and secretion. Because HDV requires the presence of HBV for replication, it is always present as part of a dual infection with HBV. Hepatitis D virus infection may occur during primary coinfection with HBV by superinfection of individuals who are chronically infected with HBV. The two viruses share common routes of transmission (Table 1). Coinfection with HDV-HBV is typically more aggressive disease, and can cause fulminant acute hepatitis, as well as rapidly progressive chronic hepatitis. The treatment of chronic HDV-HBV coinfection is challenging; interferon-α is the only available therapy, and efficacy is modest. The goal of treatment is to suppress HDV replication, and ultimately, to effect HBsAg clearance, as HDV cannot persist after HBsAg clearance. There is no preventative vaccine for HDV.

Until the early 1970s, it was assumed that there were only two types of viral hepatitis. However, once serological assays for HBV and HAV became available, cases of posttransfusion hepatitis were identified that could not be attributed to either HBV or HAV. In fact, most sera from stored samples of prospectively collected cases of posttransfusion hepatitis were sero-negative (26, 27). This new entity was labeled "non-A, non-B" hepatitis. It was another 15 years before hepatitis C virus (HCV) was identified. By extracting nucleic acid from plasma and cloning it in an expression vector, the techniques employed in the discovery of HCV heralded the development of molecular diagnostic virology (28). For the first time a pathogenic agent was identified without serology, tissue culture, or electron microscopy. HCV is a single-stranded RNA virus of the family *Flaviviridae*, and is classified into 7 genotypes. The prevalence of chronic HCV infection globally is approximately 80 to 170 million (29, 30), second only to HBV. Chronic HCV infection is a leading cause of cirrhosis and HCC in the Western world, and the most common indication for liver transplantation. The HCV-replicon system was first developed in 1999 by Lohmann, Bartenschlager and colleagues as an experimental model for HCV replication (31). Together with the crystallization of the nonstructural HCV proteins, including the NS3 protease and the NS5B RNA-dependent RNA polymerase, this allowed high throughput screening of antiviral candidates. The therapeutic developments for HCV over the past 2 decades represents one of the true success stories for translational biomedical research, starting with standard interferon-α monotherapy in the early 1990s, the combination with ribavirin (1998), the synthesis of long-acting peginterferon-α (2001), and more recently, the introduction of direct-acting antiviral agents (DAA) (2011), first in combination with peginterferon and ribavirin, followed by interferon-free combination regimens (2014). In 2016, the standard of care treatment for HCV is the combination of all oral, well tolerated and highly effective DAA combination regimens that confer cure rates ≥ 95% in clinical trials (see Chapters 13 and 54). The efficacy of these regimens is such that the WHO has proposed targets for the elimination of HCV by 2030, including 90% reduction in new cases of chronic HCV infection, 65% reduction in hepatitis C deaths, and 80% of treatment-eligible persons with chronic hepatitis C infections treated (32).

Hepatitis E virus (HEV), the most recently discovered hepatitis virus, causes an acute HAV-like illness (Table 1). The first epidemic outbreak recorded was in 1955 when 29,000 people in New Delhi developed an acute hepatitis from a water-borne source (33). It had an impressively high mortality, particularly in pregnant women in whom rates of acute liver failure were reported as high as 30%. This outbreak, as well as other similar waterborne epidemics of acute hepatitis on the subcontinent, was initially attributed to HAV, but subsequent serological testing in the early 1980s failed to implicated HAV or HBV. Further, the age distribution was older than was typical for HAV, puzzling because the Indian population is almost universally immune to HAV from childhood exposure. In 1983, Russian virologist Mikhail Balayan confirmed the existence of a non-A, non-B hepatitis transmitted via the fecal-oral route, by ingesting infected stool extracts and developing an acute hepatitis himself. He then used his own stool for electron microscopy and identified HAV-like viral particles, to which antibodies appeared during convalescence (34). However, the virus could not be grown in cell culture, and HEV was not cloned until 1990 (35). HEV is a single stranded RNA virus. Previously classified in the *Caliciviridae* family, HEV has now been classified into the *Hepeviridae* family (genus *Orthohepevirus*). Ingestion of contaminated feces is the most common route for transmission, responsible for most epidemic outbreaks, and in the West, cases are almost exclusively limited to returned travelers from endemic areas. More recently, animal reservoirs have been described, most commonly swine as well as rabbits, deer, and possibly rats. There are emerging case reports of human transmission after consumption of pork, wild boar, and uncooked deer meat in Western countries, although the rate of transmission to humans by this route, and the public health importance of this, are still unclear (36). While HEV infection almost always causes an acute, self-limited hepatitis, in immunocompromised subjects, particularly in solid organ transplanted patients, HEV may cause a chronic infection (36), with the risk of progressive liver fibrosis and cirrhosis. HEV can be particularly aggressive in pregnant women and has been associated with a high mortality rate of up to 20% due to fulminant hepatitis. There are only case reports of ribavirin being an effective antiviral agent for the treatment of chronic HEV infection (37). A recombinant HEV vaccine has been developed and was licensed in China in 2012.

Although these 5 hepatitis viruses are responsible for >95% of acute and chronic viral hepatitis, rare sporadic cases of non-A-E hepatitis continue to occur. Despite considerable effort, no novel viral hepatitis agents have been discovered in the past two decades. Candidates have included GB virus A/B, GB virus C/hepatitis G virus, TT virus, and SEN virus, but to date the data are not convincing that these agents cause hepatitis or other disease in humans.

Epidemiology

Enterically Transmitted Agents—HAV, HEV

HAV is one of the most frequent causes of food and waterborne infection worldwide and every year there are an estimated 1.5 million symptomatic cases (38). Acute hepatitis A is typically a subclinical infection of childhood. Infection in adulthood is more likely to be symptomatic, but fulminant liver failure is uncommon (0.1%), and the mortality rate of HAV is low in Western countries. However, it remains a significant cause of morbidity globally. There are distinct geographic patterns of HAV distribution, and regions of high seroprevalence are typically in the developing world, where poor sanitation, crowding, and lack of access to clean water

are common (39). Regions of high endemicity include Africa, Southeast Asia, India, and Nepal, where most children are infected before age 5. High seroprevalence rates have also been noted in South America and the Middle East, but the numbers are declining thanks to improvements in sanitation (40). Paradoxically, as sanitation improves, and fewer children are infected (40), the number of adults with protective HAV antibodies in endemic areas has declined, both increasing the risk of outbreaks due to poor herd immunity and the age-related risk of severe infection. In contrast, urbanized Asian countries such as Singapore and Japan, as well as most Western countries, have a low seroprevalence. Scandinavia boasts the lowest seroprevalence worldwide; Eastern and Southern European countries have intermediate seroprevalence, possibly a reflection of socioeconomic status. In Western populations, travel to endemic countries remains the most common risk factor for contracting HAV (41).

HEV is also enterically transmitted, either by drinking contaminated water or food. HEV is endemic in many developing countries including India and China with seroprevalences in adults of 20 to 45% (42). Outbreaks are typically seasonal and often associated with monsoon periods due to the breakdown in clean water supplies. These outbreaks can be severe, with a clinical attack rate of 1 in 2, affecting tens of thousands of people (43). The predominant genotypes in developing countries are HEV 1 and HEV 2. In industrialized, nonendemic countries, a different pattern of infection prevails, and the epidemiology of HEV is evolving. Travel to regions with high HEV endemicity remains the most common risk factor for infection, but there are recent reports of autochthonous HEV in Europe, North America, Japan, Australia, and New Zealand (36, 44). The source is zoonotic transmission from the consumption of undercooked pork, game, and offal (43), and the most prevalent genotypes are HEV 3 and HEV 4. A significant proportion of patients will have been misdiagnosed with drug-induced liver injury. In immunocompetent hosts, HEV follows the same acute, self-limiting clinical course as HAV. Adults are more likely to be symptomatic, and the average age of infection with HEV is older than for HAV. Infection during pregnancy is associated with a particularly high mortality rate at up to 30% (45). In immunocompromised hosts, HEV can persist and cause chronic hepatitis. Although uncommon, chronic autochtonous genotype 3 HEV has been reported in solid organ transplant recipients, patients with advanced HIV, and patients with hematological malignancy on rituximab chemotherapy.

Percutaneous Transmission—HBV, HCV, HDV

The transmission of HBV, HCV, and HDV is human to human, via percutaneous, sexual, or vertical transmission. There are no known animal or environmental reservoirs of the virus.

The introduction of the HBV vaccine has had a dramatic impact on the reported incidence of acute hepatitis B (AHB). The reported rate of acute HBV infection in the United States has declined since 1990, falling from 8.5 to 0.9 per 100,000 population in 2011, the lowest rate ever recorded (46, 47). Symptomatic AHB is primarily a disease of adulthood; in the same survey, the highest rates of AHB occurred among persons aged 30 to 39 years (2.0 per 100,000), and the lowest rates among persons <19 years (0.04 per 100,000). The most common risk factors were sexual exposure (multiple sexual partners, men having sex with men, and sexual contact with a person known to have HBV infection) and injecting drug use (IDU). Similar trends have been seen in other countries including Italy and Egypt, where the frequency of acute HBV infection as a cause of symptomatic hepatitis decreased from 43.4% in 1983 to 28.5% in 2002, following the introduction of childhood immunization in 1991 (48).

Between 240 to 400 million people have been estimated to have chronic hepatitis B (CHB) (49). HBV infection is the 10th leading cause of death worldwide (49), and 15 to 40% of patients with CHB develop serious liver disease, leading to 1.2 million deaths per year. Chronic hepatitis B is endemic in Southeast Asia, China, sub-Saharan Africa, Micronesia, and Polynesia, and the indigenous populations of Alaska, Northern Canada, Greenland, Australia, and New Zealand. More than 7% of the population is chronically infected in these high prevalence regions (50). Most infections are acquired early in childhood and the risk of chronicity is inversely related to the age of infection. Perinatal infection leads to chronicity in >90% cases. In contrast, infections acquired later in life tend to have a symptomatic acute phase but only a small proportion of immunocompetent patients develop chronic HBV (<5%) (51). Approximately 45% of the global population lives in an area of high prevalence. Moderate prevalence rates of 2 to 7% are seen in the Southern regions of Eastern and Central Europe, the Amazon Basin, the Middle East, and the Indian subcontinent. Low prevalence regions include much of North America, the United Kingdom, and Northern Europe, where the incidence of chronic HBV infection is less than 2%. In these countries, HBV is seen predominantly in immigrants from countries with high prevalence, and their unvaccinated offspring, as well as in specific groups with percutaneous and sexual risk factors.

Ten different HBV genotypes (A-J) have been identified, which differ by >8% of the nucleotide sequences across the genome (52). Genotype prevalence varies according to geography. Genotype A is common in Northern Europe, America, and Africa, genotype D is prevalent in the Mediterranean basis and the Middle East, whereas Asian patients are almost exclusively infected with genotypes B and C HBV. Genotypes may influence the natural history of HBV, including the timing of HBeAg seroconversion, the risk of hepatocellular carcinoma (HCC) and response to interferon-α.

Of the 240 to 400 million carriers of CHB, it is estimated that 15 million are coinfected with HDV (53). While there is a similar worldwide distribution to HBV, there is marked geographic and subpopulation variation of HDV prevalence within HBV cohorts. This discrepancy is likely due to the differences in the modes of transmission. Perinatal transmission of HDV is rare, whereas percutaneous transmission via IDU, household contact, and sexual transmission are the most common routes of infection. Coinfection is highest in the Pacific Islands, Mediterranean Europe, the Middle East, South Africa, and parts of Asia (54). In the Pacific Islands rates of coinfection have been reported of up to 90%. One survey of HBsAg positive patients in mainland China reported HDV prevalence varying from 0.8% in Sichuan 1987 to 13.3% in Guangzhou in 1990 (55). Since the introduction of the HBV vaccine there has been an overall decline in HDV. Rates in Italy have fallen from 23% in 1983 to 8.3% in 1997 (56). However there has been a recent resurgence, with the decline in Italy plateauing and rates in London, United Kingdom, rising from 2.6% in the 1980s to 8.5% in 2005 and in Germany where rates rose from 6.8% in 1997 to 8.3% in 2010 (57). While immigration from

endemic countries is partly responsible for this trend, high risk behaviors such as intravenous drug use (IVDU) and sexual practices may play a role. HDV has 4 major genotypes defined by nucleotide variation >40% across the entire genome (58); genotype 1 HDV is the most predominant in the West. The clinical relevance of HDV genotypes remains unclear.

Chronic HCV affects 130 to 150 million people globally and about 500,000 people die of HCV liver-related complications every year (59). HCV is the main cause of liver transplantation in developed countries (60). There is a significant geographic variability in prevalence. Asia, North Africa, and the Middle East are areas of high prevalence. Southeast Asia, Sub-Saharan Africa, Australasia, Latin America, and Europe have moderate prevalence, while North America and the Asia Pacific are regions of low prevalence of <1.5% (61). The transmission of HCV in developed countries is largely from IVDU, whereas in the developing world, the major route of transmission has been nosocomial through contaminated blood supply and inadequate sterilization of medical equipment, particularly unsafe injection practices. In Mediterranean countries, contaminated vaccination practices 50 years ago may explain the high prevalence in the elderly population. In Egypt, more than 10% of the population is chronically infected with HCV as a result of infection secondary to shared needles during mass antischistosomal treatment programs (62). There are six HCV genotypes, defined by sequence heterogeneity >30%, and over 50 subtypes of HCV (63). HCV genotype varies by geography also; the most common genotype globally is genotype 1 HCV. HCV genotype is very relevant clinically, as it determines the treatment regimen and likelihood of cure. Emerging data suggest that the natural history of genotype 3 HCV may be more aggressive than genotype 1 HCV (64).

PATHOPHYSIOLOGY

The pathophysiology of each hepatitis virus is complex and beyond the scope of this chapter. However, there are common features that characterize acute hepatitis. The acute injury observed in nonhepatotropic viruses such as herpes zoster, HSV-1, HSV-2, and adenovirus appears to be mediated by direct viral toxicity and the innate immune response and is characterized by necrosis of hepatocytes. In contrast, for the hepatotropic viruses, it is thought that host immunity is responsible for the acute liver injury, rather than a direct cytopathic effect of the virus. Both innate and adaptive immune responses have been implicated in hepatocyte injury as well as viral clearance, including the Toll-like receptor signaling pathway, PD-1/PD-L1 expression, NK cells, and cytotoxic T lymphocytes and regulatory T-cells. Symptomatic acute hepatitis correlates with greater intrahepatic inflammation, but the host determinants of symptomatic versus subclinical hepatitis are not fully understood (65). HAV and HEV cause a self-limiting hepatitis in immunocompetent hosts, whereas HBV and HCV may persist to cause chronic hepatitis. Age of infection is the most important determinant of HBV persistence, but the underlying mechanism is not understood. Host IL28B genotype is the most important determinant of spontaneous clearance of HCV infection (66, 67). Viral factors may however influence disease course. Fulminant liver failure is more common in the setting of acute HDV superinfection in individuals with chronic hepatitis B infection. Coinfection of HBV with HCV, or HBV with HAV, can also increase the severity of acute hepatitis. Variants of HBV, including the basal core promoter variant (A1762T/G1764A), have also been implicated in causing greater disease severity.

Hepatitis viruses may be directly cytopathic. This is rare, but may occur in immunosuppressed hosts. Fibrosing cholestatic hepatitis (FCH) due to HBV is caused by very high level HBV replication and massive accumulation of HBsAg within hepatocytes. FCH occurs in the immunosuppressed state post-liver transplantation; a similar syndrome is described involving aggressive recurrent HCV infection post-transplantation.

The histopathology of acute viral hepatitis is characterized by hepatocyte injury and necrosis, with predominantly sinusoidal and lobular mononuclear cell infiltrate, occasional neutrophils and eosinophils, and Kupffer cell hyperplasia (Figure 1) (68). Hepatocyte injury may be spotty or panlobular, and manifests as ballooning degeneration and apoptosis, with scattered acidophilic apoptotic bodies or Councilman bodies. Cholestasis is variable, and may be more prominent in HAV/HEV infection. Large hepatocytes with a "ground-glass" cytoplasmic appearance may be seen in HBV infection, with the cytoplasmic appearance reflecting accumulation of HBsAg. Hepatocellular steatosis may be present, but is normally mild in the acute setting. More prominent steatosis occurs in the setting of chronic infection with genotype 3 HCV infection as a direct viral effect. In HBV infection immunohistochemistry will demonstrate HBsAg in the cytoplasm of hepatocytes. Hepatitis B core antigen is normally found in the hepatocyte nucleus, but low concentrations may be found in the cytoplasm and on the cell membrane. The reticulin framework of the liver is preserved in uncomplicated acute viral hepatitis. In more severe cases of acute hepatitis, bridging hepatic necrosis may be seen. Bridging necrosis describes the confluent loss of hepatocytes in multiple acini, with the appearance of extensive hepatic necrosis linking venules to portal tracts. In fulminant hepatitis there is massive necrosis and dropout of liver cells in most lobules, with extensive collapse of the reticulin framework.

There is a spectrum of histological change in chronic viral hepatitis, with variable degrees of necro-inflammatory activity as well as liver fibrosis progression. Minimal necro-inflammation is confined to the portal tract. Moderate

FIGURE 1 Viral hepatitis. Top long arrow shows focal steatosis and bottom arrows show portal inflammation. Source: Prof Richard Williams, Anatomical Pathologist, St Vincent's Hospital, Melbourne.

inflammation involves piecemeal necrosis (interface hepatitis), in which the inflammatory infiltrate disrupts the limiting plate of periportal hepatocytes and extends beyond the confines of the portal tract. As the inflammation extends deeper into the liver parenchyma, lobular hepatitis develops. Severe lobular hepatitis is often accompanied by bridging necrosis. Surviving hepatocytes can cluster together forming "rosettes." There is usually no bile plugging in chronic hepatitis. Marked cholestasis is suggestive of an alternative diagnosis such as drug induced liver injury. Necroinflammatory activity drives liver fibrosis progression as part of the "wound-healing" response. Progression of fibrosis is a sequential process, involving enlargement of portal tracts, the development of periportal fibrous septae, and eventual bridging septae that connect portal tracts (bridging fibrosis). The development of intrahepatic nodules with architectural distortion is the hallmark of cirrhosis. There are a number of scoring systems for hepatic necro-inflammatory activity and fibrosis stage in viral hepatitis, including the METAVIR score, the Knodell Index, and the Scheuer score. Fibrosis can regress with treatment of viral hepatitis.

Fibrosing cholestatic hepatitis is a syndrome involving massive accumulation of viral antigens within hepatocytes. As noted, it is most common in immune-suppressed individuals post-liver transplantation. It was first described for HBV, but a similar syndrome may occur with HCV posttransplant. The pathological features include marked periportal fibrosis and cholestasis, with relatively minor inflammatory infiltrate, but widespread ballooning degeneration of hepatocytes, which likely reflects a direct cytopathic effect of the virus. Rapid progression and liver decompensation occur without treatment, but fortunately viral suppression with potent antivirals is now very effective.

CLINICAL FEATURES

Acute Viral Hepatitis

Clinical Presentation

Acute viral hepatitis presents with a characteristic syndrome that is common to all five hepatitis viruses. The spectrum and presentation of acute viral hepatitis can vary from a subclinical infection to severe fulminant liver failure. Subclinical infection is common in children, whereas adults are more commonly symptomatic. Onset of symptoms follows a variable incubation period (Table 1). Prodromal symptoms are typically nonspecific and include constitutional symptoms such as anorexia, nausea, fatigue, mild right upper quadrant abdominal pain, and low-grade fevers. Prodromal symptoms coincide with an elevation in aminotransferases and typically precede the onset of jaundice by 1 to 2 weeks. As the icteric phase begins the prodromal symptoms often settle. Clinical jaundice is associated with dark urine, pale stools, and pruritus. The liver becomes swollen and tender, with right upper quadrant pain, nausea, and anorexia. Jaundice may last for a number of weeks; biochemical tests can take longer to settle. Complete clinical and biochemical recovery normally occur in 1 to 2 months after the onset of HAV/HEV. A similar time course for clinical and biochemical recovery from icteric acute hepatitis B or C is observed when spontaneous clearance occurs; persistence and chronicity for HBV and HCV infection is defined by HBsAg or HCV RNA persistence for more than 6 months.

Beyond this stereotypical presentation, there are variations characteristic for each virus. These are best characterized for acute hepatitis A and B. HAV can have an atypical course with a prolonged cholestatic phase with the usual symptoms of pruritus, anorexia, and diarrhea caused by an accumulation of toxic mediators, primarily bile salts (69, 70). Despite a high serum bilirubin, aminotransferases can be normal during this phase. Cholestatic HAV has an excellent prognosis. HAV can also follow a relapsing course. Although acute HAV infection normally confers lifelong immunity, a small proportion of patients experience a relapsing form of the disease (71, 72). IgM anti-HAV is present, but there is evidence of viral shedding in the stool and patients are infectious. Relapses are typically mild and transient, before eventually resolving.

HAV may also be associated with extra-hepatic manifestations including a transient rash and arthralgia. Clinical manifestations of immune complex formation (involving IgM anti-HAV) are uncommon but include vasculitis, glomerulonephritis, cryoglobulinemia, and blood dyscrasias. Several reports describing an episode of HAV immediately prior to the onset of autoimmune hepatitis (AIH) suggest a possible association between HAV and AIH, although causality has not been established.

The majority of acute HBV infections are clinically silent, especially in children. Icteric hepatitis is more common in adults. The disease tends to be more severe if the patient is coinfected with another hepatitis virus or has underlying liver disease. Chronicity of HBV infection is determined by the age of infection, size of the inoculum, and immune response of the host. As noted, age of infection is the most important determinant of persistence. Extrahepatic manifestations of HBV infection include the serum sickness-like prodrome of acute HBV infection, polyarteritis nodosa, HBV-associated glomerulonephritis, mixed essential cryoglobuliemia, and neurological manifestations and are also thought to be mediated by circulating immune complexes (73). A serum sickness-like prodrome precedes acute HBV infection by 1 to 6 weeks in 10 to 30% of cases. Also known as the "arthritis-dermatitis" syndrome, it is characterized by a symmetrical generalized inflammatory arthritis, typically involving the small joints of the hands and feet. The joint lesions are nondestructive. Fever is common. Skin manifestations are variable, occurring in more than 50% of those with joint symptoms. Lesions described include maculopapular, petechial or purpuric rash, palpable purpura, Henoch-Schönlein-type purpura, erythema multiforme, toxic erythema, lichenoid dermatitis, and urticaria. Renal involvement with proteinuria or hematuria is much less common. Angioneurotic edema may rarely occur. Polyarteritis nodosa (PAN) is a rare but serious complication of HBV infection. The syndrome normally presents within 4 months of the clinical onset of HBV infection, with abdominal pain due to arteritis of medium-sized vessels causing ischemia of the intestine and gallbladder. Angiography demonstrating microaneurysms of blood vessels in the renal, hepatic, or mesenteric circulations is virtually pathognomonic. Tissue biopsy of affected organs reveals inflammation of the medium-sized arteries. The prognosis is poor without treatment, with mortality of up to 50%. Glomerulonephritis (GN) and other HBV-related manifestations are more common in the setting of chronic HBV infection and will be discussed below.

Fulminant Viral Hepatitis

Fulminant viral hepatitis is the most severe form of acute hepatitis presenting as acute liver failure (ALF). ALF is defined by the development of severe acute liver injury with encephalopathy and impaired synthetic function

International Normalised Ratio (INR) of ≥ 1.5) in a patient without cirrhosis or preexisting liver disease. ALF is often associated with multisystem organ failure, disseminated intravascular coagulation, and it is often complicated by sepsis and cerebral edema. Patients with ALF should be managed by a liver transplant service in the intensive care unit. The mortality rate for ALF very high, but outcome post-liver transplantation is good. Viral hepatitis is the most common cause of ALF in developing countries (74) and develops in approximately 0.3% of HAV patients and in approximately 0.5% of HBV patients. It is more common in adult-onset infection and when infection occurs in an individual with premorbid liver disease; e.g., acute HAV infection in a person with chronic hepatitis C, especially if cirrhotic. Coinfection of super-infection with HDV confers a significant risk for ALF and testing for delta virus is recommended in all cases of HBV-related ALF (75). It is very rare for HCV to be associated with ALF. Where HCV is associated with ALF, it is normally in the setting of HBV or HAV coinfection. The majority of patients with fulminant HCV are coinfected with HBV. HEV is an important cause of ALF in endemic areas with a fatality rate of up to 25% in pregnant women, especially in the third trimester (76). HSV in pregnancy, although rare, is also associated with a fulminant course with high mortality and morbidity. Timely administration of acyclovir can reduce the risks to mother and infant (77, 78).

Liver Function Tests

All forms of acute viral hepatitis cause biochemical abnormalities in liver function tests. Typical laboratory abnormalities include marked elevations of the aminotransferases, alanine aminotransferase (ALT), and aspartate aminotransferase (AST), which indicate hepatocellular injury. Serum ALT is usually higher than AST in viral hepatitis. Bilirubin also rises acutely and may continue to rise even after the aminotransferases have started declining. Once bilirubin exceeds approximately 3 mg/dl (50 micromol/l), jaundice becomes clinically apparent, with yellow pigmentation of the sclera and dark urine. The titer of serum aminotransferases and bilirubin can be quite impressive, but they are not markers of severity or prognosis. Coagulopathy is a marker of fulminant hepatitis (see below). Fulminant hepatitis is indicated by a loss of synthetic function acutely with prolongation of the prothrombin time.

It is important to recognize that a presentation with acute hepatitis on clinical and biochemical grounds is not specific to viral hepatitis. Important differential diagnoses to consider for acute hepatitis include systemic viral infections, drugs and toxins, ischaemia, autoimmune hepatitis, Wilson's disease, Budd-Chiari Syndrome, and pregnancy-related liver disease amongst others (see Table 4). In children, rubella has been associated with hepatitis. In returned travelers, the hemorrhagic fever viruses (including Ebola, Marburg, Lassa viruses) and yellow fever (group B arbovirus) have been associated with hepatitis as part of a severe systemic illness.

The pattern of abnormalities of laboratory tests may be suggestive of a diagnosis but imagining and specific tests are required to confidently establish the etiology.

Virus-Specific Serologic Features

The acute viral hepatitides can be differentiated on the basis of serology (Table 2).

HAV

HAV is diagnosed based on the presence of anti-HAV antibodies in the serum. The gold standard diagnostic for acute HAV infection is the presence of anti-HAV IgM, which, in presence of the features of an acute hepatitis, is sufficient to confirm the diagnosis. Anti-HAV IgM is positive by the time of symptom onset, peaks during the icteric phase and remains positive for approximately 6 months. Anti-HAV IgG appears in the convalescent phase, remains detectable for decades, and correlates with immunity against further HAV infections. HAV can also be detected in fecal samples by electron microscopy or measuring HAV RNA but

TABLE 2 Common serological patterns of viral hepatitis

Virus	Serologic pattern	Interpretation
HAV	IgM anti-HAV+	Acute infection
	IgG anti-HAV+	Past infection
HBV	HBsAg+, IgM anti-HBc+	Acute infection
	HBsAg+, IgG anti-HBc+, HBeAg+, HBV DNA +	Chronic, replicative infection
	HBsAg+, IgG anti-HBc+, HBeAg-, anti-HBe+ HBV DNA $>10^4$ copies/ml	Chronic, replicative infection with precore or core promoter mutant
	HBsAg+, IgG anti-HBc+, HBeAg-, anti-HBe+ HBV DNA $<10^4$ copies/ml	Chronic, minimally replicative infection
	HBsAg-, HBV DNA+	Occult HBV
HDV	Anti-HDV+, HBsAg+	HDV infection
	HBsAg +, IgM anti-HBc+, low titer anti-HDV	Acute coinfection
	HBsAg+, IgG anti-HBc+, HDV RNA +, rapidly increasing titers anti-HDV	Acute superinfection
	HBsAg+, IgG anti-HBc+,HDV RNA+, High titers anti-HDV	Chronic HDV
HCV	Anti-HCV+, HCV RNA+	HCV infection
	Anti-HCV+, HCV RNA-	Past infection or false positive antibody
HEV	IgM anti-HEV+	Acute infection
	IgG anti-HEV+	Past infection

these remain research tools. Serology is the simplest and most convenient test available and is therefore the gold standard.

HBV

The standard serology panel for diagnosis of HBV infection, past or present, should include HBsAg, anti-HBs, and anti-HBc. Hepatitis B surface antigen (HBsAg) is the first serologic marker to appear at approximately 1 to 10 weeks after inoculation. HBsAg precedes the onset of biochemical or clinical abnormalities. In patients who achieve spontaneous clearance, HBsAg will decline to be undetectable at 6 months. HBsAg persisting beyond the 6-month mark is by definition chronic HBV infection. HBsAg levels can be quantified, and in the setting of chronic hepatitis B, have been correlated with HCC risk as well as interferon-α treatment response (see below) (79). Resolution of HBV infection is marked by the appearance of anti-HBs antibodies in the serum. There may be a lag of weeks to months between the clearance of HBsAg and the appearance of anti-HBs (the serological "window" period). During this window period, the only serological evidence of HBV infection is the presence of antibodies against hepatitis B core antigen (IgM anti-HBc). Anti-HBs antibodies also become detectable following vaccination; protective immunity following the standard 3 dose vaccine course is indicated by and anti-HBs titre >10 IU/ml.

HBV core antigen (HBcAg) forms the capsid of the virus, and is found in infected hepatocytes rather than serum. However, antibodies to HBcAg, or anti-HBc, are detectable in serum approximately 1 to 2 weeks after the appearance of HBsAg. IgM anti-HBc appears first. The presence of anti-HBc IgM, especially at high titer, is a marker of acute HBV infection, and can help differentiate true acute infection from a relapse of chronic HBV infection. For the first 6 months of infection, the predominant class of anti-HBc is IgM. As noted, anti-HBc may be the only serological marker of acute HBV infection during the window period. Titers of anti-HBc IgM decline over 6 months, after which IgG appears and becomes the predominant class of anti-HBc. Anti-HBc persists long term. Anti-HBc is a marker of HBV exposure. Anti-HBc differentiates immunity due to past infection from vaccine-induced immunity. Anti-HBc may be the only marker indicating past infection in older patients as anti-HBs levels wane. Testing for anti-HBc is particularly important when screening patients for HBV prior to immunosuppression. Isolated anti-HBc has been reported in approximately 2% blood donors in areas of low endemicity but approximately 10 to 20% in areas of high prevalence and in the HIV population (80–82). There is a risk of fulminant HBV reactivation in people who are HBsAg negative, anti-HBs negative, but anti-HBc positive (e.g., in the setting of the anti-CD20 monoclonal antibody rituximab).

The hepatitis B "e" antigen (HBeAg), or precore protein, is a secreted, soluble protein that can be detected in serum. Although it not necessary for viral replication, HBeAg is believed to act as a tolerogen to attenuate the immune response to HBV and promote viral persistence. HBeAg is considered a surrogate marker of HBV replication and infectivity. HBeAg is almost always present during acute HBV infection. Seroconversion to anti-HBe occurs with viral clearance as the HBV DNA falls, and often precedes the appearance of anti-HBs. However there are viral variants defective for the HBeAg, the most common carrying a G1896A precore mutation which abrogates HBeAg production. This variant is commonly selected in the course of chronic hepatitis B, emerging as an immune escape variant to cause HBeAg-negative CHB. Testing for HBeAg provides limited clinical information in the acute setting, but is important for patients diagnosed with chronic hepatitis B as it helps identify the phase of infection as well as the emergence of immune escape variants.

Polymerase chain reaction (PCR) testing for serum HBV DNA can be positive in patients as early as 10 days post-infection. Recovery from HBV is associated with clearance of HBV DNA from the serum. As for HBeAg, measuring HBV DNA levels in the setting of acute HBV infection provides little extra information and is not routine. In the setting of chronic hepatitis B, HBV DNA levels are very important for prognostication and monitoring treatment response.

HCV

The screening test for HCV infection is serology, and anti-HCV antibodies are detectable in the majority of patients at presentation, becoming positive 4 to 10 weeks following infection (Table 1). Anti-HCV indicates exposure, but remains positive following spontaneous clearance. Therefore, diagnostic testing for HCV includes serology as a screening test followed by confirmatory testing with molecular assays that quantify viral load (HCV RNA). Anti-HCV can be falsely negative early in acute HCV as the antibodies can take 2 to 6 months to appear. Serum HCV RNA is more sensitive and becomes detectable as early as 10 days postinfection.

HDV

HDV depends on HBV coinfection, so HBsAg should be included in any testing algorithm for HDV. HDV antigen (HDAg) appears early but is short-lived as circulating anti-HDV interferes with the assay. Testing for anti-HDV is more reliable. As for HCV, serology for HDV should be considered a screening test, to be confirmed by molecular virology. Although there are no licensed assays for serum HDV RNA, most specialty laboratories will offer an in house assay. It is likely that there will be an approved assay in the near future. The differentiation of acute HDV/HBV coinfection vs HDV superinfection is most commonly made on clinical grounds. The pattern of anti-HBc serology may help; detectable anti-HBc IgM is suggestive of acute coinfection, while anti-HBc IgG suggestions superinfection. Acute coinfection typically presents as a severe acute hepatitis that then resolves. The majority of HDV superinfections (>70%) become chronic.

HEV

The routine screening test for the diagnosis of HEV is serology. IgM anti-HEV appears early before waning over months. IgG appears shortly after IgM and persists for years after the illness, providing protective immunity. Antibody tests in HEV are not optimal as they are associated with frequent false positive and negative results. Moreover the assays can be variable depending on which commercial testing kit is used (83). HEV RNA testing of serum or stool is offered by research laboratories, and can considered when there is diagnostic doubt. HEV RNA testing must be performed early in the disease course. HEV can be detected in the stool about a week before symptoms appear and until two weeks after resolution of the infection. Fecal shedding implies infectiousness.

Chronic Viral Hepatitis

HBV and HCV are the most common causes of chronic viral hepatitis. Most patients with chronic hepatitis B or

TABLE 3 Screening for HBV and HCV is recommended in people with the following risk factors

People who inject drugs
People with a history of tattooing or body piercing
People in custodial settings
Sex workers
Sexual partners of an HCV-infected person should be tested for HCV
Partners and other household and intimate contacts of people who have acute or chronic hepatitis B infection should be tested for HBV infection
Children born to HCV- or HBV-infected mothers
Pregnant women
People who received a blood TF/organ Tx prior to 1990
People infected with HIV or HBV/HCV, respectively
People with evidence of liver disease (persistently elevated ALT level)
People who have had a needle-stick injury
Migrants from high prevalence regions (Egypt, Pakistan, Mediterranean and Eastern Europe, Africa, and Southern Asia)

hepatitis C are asymptomatic. Screening strategies targeted to high-risk individuals are important (Table 3). The common symptoms attributed to chronic hepatitis are malaise, fatigue, and anorexia. The long-term hepatic complications of viral hepatitis include progressive liver fibrosis, cirrhosis, liver failure, and hepatocellular carcinoma. Chronic hepatitis B is associated with a particularly high risk of HCC, which may arise in the noncirrhotic liver. Viral hepatitis is the most common cause of HCC globally, and a major cause of global mortality. The clinical features and natural history of chronic hepatitis B and C are discussed in detail in Chapters 32 and 54, respectively.

Extrahepatic manifestations of chronic viral hepatitis warrant consideration here. They are an indication for antiviral therapy. Glomerulonephritis (GN) is commonly associated with CHB. The most common presentation is nephritic syndrome. A number of patterns of glomerular injury have been described, including membranoproliferative GN, membranous GN, and rarely mesangial proliferative GN. In children the disease is usually self-limited; however, progression to renal failure has been described in adults. The association of HBV infection with mixed essential cryoglobulinaemia is controversial. Rare cases have been reported; however, the majority of cases are now recognized to be associated with HCV infection (73).

CHC is also associated with extra-hepatic manifestations. Mixed cryoglobulinemia is a systemic problem that results in the deposition of immune complexes in small and medium blood vessels. It is common in HCV and typically presents as a purpuric, vasculitic rash usually on the shins, arthralgia, and glomerulonephritis. HCV can be associated with a membranoproliferative glomerulonephritis and membranous nephropathy (84). HCV is also associated with autoimmune disorders including immune thrombocytopenic purpura, autoimmune hemolytic anemia, thyroid problems, and myasthenia gravis.

Role of Liver Biopsy

Liver biopsy is now rarely performed in patients with viral hepatitis. Liver biopsy is not usually necessary for either the diagnosis of acute hepatitis or for differentiating etiology. Liver biopsy is indicated when there is diagnostic doubt. Liver biopsy was used for many years to stage liver fibrosis in patients with chronic viral hepatitis, for determining prognosis, and identifying treatment candidates. However, the recent introduction and wide availability of noninvasive markers for liver fibrosis, including transient elastography and serum biomarkers, has largely replaced histology for staging liver fibrosis. Liver biopsy is reserved for those patients in whom the results of less invasive techniques are unclear, and when clinical decision-making will be directed by the result of the biopsy.

SPECIAL POPULATIONS

Transmission in Healthcare Workers

Healthcare workers are at risk of infection from blood-borne pathogens including HBV, HCV, and HIV through occupational exposure to blood products and body fluids. All body fluids and tissue should be considered potentially infectious. Needle stick injuries from contaminated needles are the most common route of transmission in the healthcare setting, but transmission can also occur through exposed mucosa or abraded skin. The risk of acquiring HBV following occupational exposure depends on the serum HBV DNA level and HBeAg status of the source (85). HBV is highly infectious; the risk of transmission is 20 to 60% depending on the HbeAg status of the source. HBV transmission in healthcare workers is dropping thanks to routine recommendations for HBV immunization among healthcare workers (86). The risk of HCV transmission is significantly less than HBV, of the order of 1 to 4% (87), but the risk of chronicity is high. Unfortunately there is no preventative vaccine for HCV. Risk minimization and management should be a central part in occupational health and safety programs, as well as the infection control plans of any healthcare facility. This includes staff education, the use of protective equipment, sharps disposal facilities, universal HBV vaccination, and postexposure prophylaxis. Standard precautions including hand hygiene and the use of gloves, gowns, and protective eyewear should be strictly adhered to.

Following exposure, wound care with flushing of the site should be immediately instituted. If the healthcare worker has evidence of anti-HBs, either from previous exposure or vaccination, postexposure management is not required. If there is no preexisting protection against HBV, hepatitis B immunoglobulin (HBIg) should be administered as soon as possible, which can reduce the risk by 75% (88). HBIg should be administered concurrently with the first dose of the HBV vaccine. There is no postexposure prophylaxis for HCV. The recent development of oral highly effective antiviral therapy for HCV is likely to lead to studies of the use of these agents for postexposure prophylaxis, particularly in endemic areas, or in healthcare workers who might face exclusion from the workplace pending diagnostic evaluation.

Hepatitis in Pregnancy

The most common cause of jaundice in pregnancy globally is viral hepatitis (89). Acute HAV during pregnancy is associated with preterm labor, but the clinical course is otherwise unremarkable. HAV is not vertically transmitted. The HAV vaccine is safe in pregnancy and should be administered to pregnant women traveling to endemic areas (103). Similarly HAV immunoglobulins are safe and can be administered following exposure to HAV. HEV in pregnancy can lead to

TABLE 4 Differential diagnoses for acute liver injury

Differential diagnosis	Clues on diagnostic tests
Systemic infections • Herpes Simplex Virus (HSV-1, HSV-2) • Cytomegalovirus (CMV) • Epstein Barr Virus (EBV) • Varicella Zoster virus (VZV) • Parvovirus B19	Markedly elevated transaminases, leukopenia, low bilirubin
Drugs/Toxins • Acetaminophen • Idiosyncratic drug reactions • Herbal preparations • *Amanita Phalloides* mushroom	Very high enzyme elevation, low bilirubin
Ischaemic hepatitis	Very high aminotransferase levels (25 to 250 times the upper limit of normal), elevated serum LDH levels
Autoimmune hepatitis	Auto-antibodies (ANA, ASMA, ALKM-1)*
Wilson's disease	Coombs-negative hemolytic anemia, aminotransferase levels < 2,000 IU/l, AST to ALT ratio of > 2, normal or markedly subnormal ALP (< 40 IU/l), ALP to total bilirubin ratio < 4, low uric acid levels
Veno-occlusive disease • Budd-Chiari • Portal vein thrombosis	Doppler ultrasound
Pregnancy-related liver failure • Acute fatty liver of pregnancy • HELLP syndrome	Aminotransferase levels < 1,000 IU/l, elevated bilirubin, low platelet count

*ALP: Alkaline phosphatase, ANA: Anti-nuclear antibody, ASMA: Anti smooth muscle antibody, ALKM-1: Anti liver kidney micosomes.

fulminant liver failure with a high mortality of up to 25% in the third trimester. It is a major cause of maternal and fetal mortality in areas of high endemicity such as India, China, and Africa. HEV infection in pregnancy not only carries a high maternal mortality risk, but is also associated with a higher rate of obstetric complications and poor fetal outcomes including spontaneous abortion, stillbirth, preterm delivery, and antepartum hemorrhage (90). A high rate of vertical transmission has been reported in HEV (91) especially if the maternal viral load is high. There is no current treatment for HEV in pregnancy. Ribavirin is teratogenic and is therefore contraindicated in pregnancy and there is no evidence of efficacy in the pregnant patient. There are no safety or efficacy data for the new HEV vaccines in the pregnant population.

Acute HBV during pregnancy is usually not severe as pregnancy is a state of immunosuppression. There have been reports of prematurity and low birth weight, but there is no increased risk of mortality or teratogenicity. In chronic HBV, HBV DNA levels tend to be stable throughout pregnancy. HBV may flare in the postpartum period because of immune reconstitution and HBe seroconversion can occur during those flares. The perinatal transmission rate in untreated HBeAg positive mothers is approximately 90% (92). Most infections appear to occur at the time of delivery. HBV screening during pregnancy and universal vaccination of all newborns has reduced transmission rates. Prophylactic administration of HBIg at birth followed by routine vaccination reduces transmission rates to 5 to 10%. The most important risk factor for vertical transmission is the mother's viral load. Antiviral therapy is now recommended in the third trimester for mother's with a serum HBV DNA level $> 10^6$ IU/ml (93). The baby should still receive HBIg and vaccine immediately postpartum. The nucleotide analogue, tenofovir is a Category B drug that can be used safely in pregnancy. Lamivudine is classified a Category C but it has been used extensively in the HIV population with a reasonable safety profiles. Normal vaginal delivery is recommended as Cesarean delivery does not reduce the risk of infection (94); breastfeeding is safe (95).

Vertical transmission of HCV is much less efficient than of HBV, but the ~5% risk is higher in the setting of HIV coinfection (96), in which the viral load is higher (97). Most pregnancies are uncomplicated although a lower birth weight has been reported. As with HBV, normal vaginal delivery and breastfeeding are still recommended (98). Newborns will test positive for anti-HCV as they automatically acquire maternal antibodies so they need to be tested for HCV RNA.

Chronic viral hepatitis with cirrhosis causes a unique set of challenges for pregnancy. Cirrhosis is associated with infertility; those who become pregnant have a variable course. There is a risk of hepatic decompensation. The risk of poor fetal outcomes including miscarriage, stillbirths, and preterm labor is increased. Portal hypertension may worsen in pregnancy due to the increase in total blood volume and the risk of variceal hemorrhage is significant. Screening for varices and prophylactic banding should be considered prior to pregnancy.

Viral hepatitis is not the only cause of liver problems in pregnancy. Differential diagnoses unique to pregnancy must be also be considered during assessment and include hyperemesis gravidarum, acute fatty liver of pregnancy, intrahepatic cholestasis of pregnancy, and preeclampsia/HELLP syndrome (a variant of preeclampsia presenting with hemolysis, thrombocytopenia, and liver disease). Symptomatic gallstones are common in pregnancy.

Viral Hepatitis in the HIV Population

With shared transmission risks, the prevalence of chronic hepatitis B and hepatitis C is higher in the HIV-infected population. With improved control of HIV disease with antiretroviral therapy, liver disease has emerged as one of the leading causes of death in patients with HIV (30). HIV impacts directly on the outcome of HCV and HBV infection, complicating its natural history, diagnosis, and management. For reasons that remain unclear, liver damage, especially fibrosis, progresses at a faster rate than in HCV or HBV mono-infection, despite hepatic necro-inflammation typically being less severe. Levels of HCV or HBV viremia tend to be higher. This is possibly due to increased replication in the setting of the relative immunosuppressive effect of HIV or a direct effect of the HIV itself that facilitates the engagement of coreceptors (99). Therefore, all patients with HIV should be screened for HBV and HCV infection. It is recommended that testing for both anti-HBc and HBsAg be

performed, as patients with HIV can have occult HBV, with high levels of HBV DNA and anti-HBc, but not HBsAg. Similarly, all HBV/HCV patients should undergo HIV testing (24). In the past, treatment of both HBV and HCV has been less effective in HIV coinfected patients. This is no longer the case with potent first line direct DAAs, and response rates are generally equivalent to those observed in the mono-infected population. The management of drug-drug interactions is more complicated in patients taking HAART and there is a risk of hepatotoxity. Flares of hepatitis due to immune reconstitution have also been reported (100, 101). However the benefit of HAART is thought to outweigh the risk as it is associated with a slower rate of liver fibrosis (102). HCV/HIV and HBV/HIV coinfection will be considered in further detail in separate chapters.

Hepatitis in Patients Undergoing Immunosuppressive Therapy

Screening for HBV and HCV infection is recommended for all patients being considered for immunosuppressive therapy. Monoclonal anti-CD20 therapy, and stem cell transplantation are particularly high risk. Immunosuppression is associated with risk of severe hepatitis flares in patients with chronic hepatitis B infection. This can be prevented by the use of nucleos(t)ide analogue therapy. In patients with chronic hepatitis C, immunosuppression is associated with higher levels of serum HCV RNA and more rapid fibrosis progression; this can be prevented by curative antiviral therapy. Specific details regarding recommendations for testing and treatment are considered in pathogen-specific chapters.

Liver Transplantation in Viral Hepatitis

HBV and HCV are common causes of cirrhosis and HCC and remain the main indications for liver transplantation. HCV is the most common indication for liver transplantation. Following liver transplantation for HCV, graft recurrence has been universal, and the progression to fibrosis in the graft may be rapid due to the concurrent use of immunosuppressants. There is also a risk of fibrosing cholestatic hepatitis However, these events can now be prevented by complete HCV suppression with DAA therapy pretransplant. New DAA therapies for HCV are also very effective posttransplant, and can prevent rapid fibrosis progression, as well as effectively treat FCH. Graft recurrence with HBV is also universal and requires posttransplant prophylaxis with nucelos(t)ide analogue therapy and HBIg. The prognosis post-liver transplantation for patients with chronic hepatitis B is excellent.

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Viral Infections in Organ Transplant Recipients

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6

Solid organ transplantation (SOT) and hematopoietic cell transplantation (HCT) represent continually expanding fields of medicine, and, with many innovative methods for allograft management, new and unusual presentations of virus infections continue to occur. These new drugs or modalities aim to protect the SOT recipient from rejection of the newly acquired organ by the endogenous immune system or to protect the recipient from attack by the graft (Graft vs. Host Disease, GVHD). For example, in the mid-1960s, with the introduction of cytotoxic drugs such as azathioprine and cyclophosphamide in renal transplantation, pneumonitis associated with human cytomegalovirus (CMV) infection was first observed (1). Soon thereafter, it was noted that transplant recipients with Epstein-Barr virus (EBV) infection developed a previously unrecognized clinical syndrome, posttransplantation lymphoproliferative syndrome (PTLD) (2). In populations with a high prevalence of human herpesvirus 8 (HHV-8) infection, Kaposi sarcoma became a problem following SOT (3). With time, most of the endogenous herpesviruses and polyomaviruses of humans have emerged as particular problems. At the same time, respiratory viruses and hepatitis viruses complicate successful management of the SOT and HCT recipient as methods of iatrogenic immunosuppression change. The donor tissue itself can be the source of transmission of virus infection, including rabies (4), West Nile virus (5), human T leukemia virus type I (6), human immunodeficiency virus (7), lymphocytic choriomeningitis virus (8), and B19 parvovirus (9).

Complex factors determine when and which active infection will occur and whether it will progress to disease. The time of onset of the most common viral infections after transplantation is fairly predictable (Fig. 1), and during the first year, infections with CMV, EBV, HHV-6, HHV-8, and BK virus (BKV) become a major focus of attention for the clinician. Consequently, management of these virus infections will be emphasized in this chapter. Correct management of hepatitis B virus and hepatitis C virus infections (Chapters 32 and 54) and of the several respiratory virus infections (Chapters 27, 37, 43) are equally important in patient outcome, but are discussed elsewhere in this volume.

CYTOMEGALOVIRUS INFECTION

Epidemiology, Risk Factors, and Pathogenesis of CMV Disease

Epidemiology

Human cytomegalovirus (CMV) (Chapter 23) was initially identified, and, in fact, was named because of its effect on the fetus (10), but the virus has come to be recognized as a major pathogen in the organ-transplant recipient (11). Beyond the newborn, CMV produces disease almost exclusively in the immunologically impaired individual, and, in doing so, it demonstrates considerable variability in the types of clinical syndromes that are seen in different at-risk groups. Thus, in the fetus and in the child with perinatal infection, there can be hearing loss, central nervous system disease, and retinal damage (12). In SOT recipients, chronic CMV infection produces mononucleosis-like syndromes, arthralgia, enteritis and hepatitis, and pneumonitis (13, 14). With the introduction of preventive antiviral strategies, there has been a significant change in the occurrence of life-threatening CMV infection and in the management of transplant patients. In the 1980s, the CMV-related mortality was 10 to 30% for recipients of allogeneic HCT (15), but, with the use of ganciclovir, mortality has dropped to 2 to 6% in this population (14, 16, 17).

The use of antiviral chemotherapy has altered the natural course of posttransplantation CMV. In the pre-antiviral era, the onset of disease occurred at 2 to 3 months post transplant, but with the use of antiviral suppression, CMV reactivation and disease onset is delayed and occurs in the 4- to 12-month period post transplant (16). Of note, however, in the HCT population, the best antiviral agents have not been able to prevent late CMV disease (18). In a review of 9,469 recipients of allogeneic HCT between 2003 and 2010, CMV reactivation remained a significant risk factor for lower overall survival (17). Thus, with a shift in disease onset to later times after transplantation and with the side effects of antiviral treatment, the strategies for management of the transplant recipient have become more complicated. Treatment plans must weigh these risks, including costs, against the anticipated benefits and develop a rational

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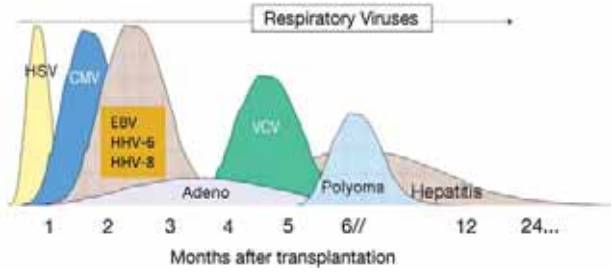

FIGURE 1 Occurrence of the most frequent virus infections after transplantation. The time after both solid organ and marrow transplantation correlates with characteristic types of virus infections, ranging from herpes simplex virus (HSV) at the earliest time, to cytomegalovirus (CMV), Epstein-Barr virus (EBV), herpesvirus type 6 (HHV-6), and herpesvirus type 8 (HHV-8) at 1 to 3 months after transplantation and with adenovirus, varicella zoster virus (VZV), polyomavirus, and hepatitis viruses at later times.

approach. Thus, the person with responsibility for the care of transplant recipients must be familiar with the changing aspects of CMV infection and prevention.

Risk Factors for CMV Infection in the Allograft Recipient

The management of HCT and SOT recipients is based on patient risk factors, and, in order to better understand the biologic aspects of CMV infection and to minimize CMV complications in these patients, it is important to recognize these factors (Table 1). The most significant one is the development of CMV infection itself, and for that reason, patient age >20 years, a prior infection of the donor with CMV, and a prior infection of the recipient with CMV are most important. Early sero-epidemiologic (11), as well as molecular (19), evidence indicated that the donated organ is the source of CMV reinfection in most instances. Primary CMV infection can occur, but, more frequently, reinfection occurs in the recipient who has had prior CMV infection and acquires a new CMV strain from the donor (19). Cadaver organ transplants are also associated with more CMV infection than are organs from living donors (20). Thus the serostatus, defined as positive (+) or negative (-) CMV antibody in serum, of the donor (D) and the recipient (R) are used to group patients by risk. D+/R- SOT transplants are at highest risk of CMV infection and disease (21). Without preventive measures, 80 to 100% of D+/R- recipients will develop CMV infection, and 50 to 70% will develop disease (22). But the risk for D+/R+ recipients is reduced, and the risk for a D-/R- transplant is nil. Next in the hierarchy of risks for CMV-related complications are the type of organ, the age of donor and recipient, the type and intensity of immunosuppression, the time since transplant, coinfections, and presence of organ rejection (23). Regarding the type of organ, lung, small bowel, pancreas, and combined kidney-pancreas SOT recipients are at the highest risk for CMV infection (13, 24). Liver and lung recipients are at intermediate risk, and kidney recipients are at lowest risk (13, 24). Absolute CMV load, as measured by PCR in plasma, has also been associated with risk for CMV disease (25). In this regard, the CMV load is usually higher in association with more potent immunosuppression. However, with the use of an m-TOR-based immunosuppressive regimen, such as sirolimus or everolimus, there is a decreased incidence of CMV infection and disease (26). One study, comparing sirolimus-mycophenylate mofetyl-corticosteroid versus tacrolimus-mycophenylate mofetyl-corticosteroid, found a decrease in CMV infection (3% vs. 12%, $P = 0.02$) with no difference in rejection rates (27, 28). The presumed explanation for this is that m-TOR inhibitors regulate CD8 memory T-cell development and enhance both the quantity and quality of the immune response (26). Also, sirolimus has been reported to inhibit CMV replication *in vitro* (29). In both the SOT and HCT transplant recipient, age is a risk factor; the pediatric recipient is more likely to be R- and subject to CMV infection on this basis depending on the donor serostatus. Finally, the induction of immunosuppression with antilymphocyte therapy is particularly associated with CMV infection, and any added immunosuppression for organ rejection or GVHD increases the risk for CMV infection (30).

In considering risk factors of CMV disease after transplantation, immunosuppression is, in fact, considerably important. Patients who have detectable cytotoxic T-lymphocyte (CTL) activity targeted to CMV have significantly less disease than those without such CTLs (31–33). In addition, the absence of CMV-specific $CD4^+$ T-cell function is a marker for CMV disease (34, 35). The frequency of CMV-specific CD4 and $CD8^+$ T cells can be assessed and evaluated quantitatively (36), and studies have linked the absolute numbers of CMV-reactive T cells to protection from disease (37–39). In HCT, the posttransplant reacquisition of CMV-specific cellular immunity following marrow ablation is improved when the donor has prior immunity to CMV (40). Similarly, in solid organ transplantation, the influence of cellular immune modification by the use of anti-T-cell antibody or of other agents that can influence CTLs, will significantly increase the risk of progressive CMV infection (20, 41).

TABLE 1 Risk factors for CMV complications in the transplant recipients

CMV Infection
Seropositive donor/seronegative recipient (D+/R-)
Graft as source of infection
Age
Mismatch Status of Solid Organ Donor
Mismatch number
HLA-type: DR7, DRw6, B7
Organ Type
Liver, Heart, Lung > renal transplant
Cadaver > living donor solid organ transplant
Allogeneic > autologous marrow transplant
Immune Status
Immunosuppressive regimen
CMV-specific lymphocyte immunity

Pathogenesis of CMV Infection after Allograft Transplantation

CMV-associated diseases in transplant populations are acquired through transmission of infection from the transplanted organ or from reactivation of endogenous infection in the recipient (11, 19). As with other herpesviruses, CMV can reactivate from a latent infection and lead to persistent infection that progresses to organ-specific disease if there is an absence of functional CMV-specific cell-mediated immunity (16). Local recovery of CMV at sites, such as urine or throat, do not correlate with incidence of disease, whereas

CMV viremia and asymptomatic pulmonary infection strongly correlate with subsequent disease (13, 14). It appears, then, that progression of CMV from local to disseminated infection is necessary before the onset of serious CMV disease in the setting of immunosuppression.

As with CMV infection in the non-transplant setting, the syndromes that occur with CMV infection in the transplant recipient can range from severe disease to mild forms of mononucleosis. For example, encephalitis, enteritis, pneumonitis, and other organ-specific syndromes occur, as seen in AIDS and in neonatal CMV infection (42, 43). However, the usual course includes fever, malaise and fatigue similar to infection in healthy adults (44). The usual asymptomatic course of CMV infection of normal persons is also seen with CMV infection in some transplant recipients (45, 46). Interestingly, although the rates of infection with CMV and the timing of infection post transplant are nearly identical when renal, heart/lung, and marrow transplant populations are compared, the severity of disease is much greater in heart/lung and HCT patients than in renal transplantation. However, the neurotropism of CMV infection, seen in AIDS and in the fetus and young infants, is less frequently seen in HCT and SOT recipients (13, 41, 46, 47). The principal differences in presentation of CMV disease between these groups are the predominance of mononucleosis-like symptoms in the HCT patients and direct involvement of the transplanted organ in the case of SOT (13, 41, 48).

In addition to direct effects of viral infection on organ function, CMV infection after transplantation is associated with events such as graft rejection, atherosclerosis, and increased rates of bacterial and fungal infections (13, 49–51). Because these effects have been associated with immunological abnormalities specific for transplantation populations, such as host-versus-graft disease or GVHD, the symptom complex associated with CMV appears to be due not only to a direct cytotoxic effect of infection on cell or organ function but also to a secondary or indirect effect on host responses to infection. Therefore understanding the pathogenesis of CMV disease in the transplant recipient involves elements related to (a) onset and progression of virus infection and (b) the effect of this infection on general cellular function and on specific immune function.

Monitoring the Immune Status of the Patient

Assays for Nonspecific Monitoring on Immune Status

In the ideal world, a transplant physician would be able to monitor the immunologic status of the patient and tailor immunosuppressive therapy to the needs of the patient based on risk and organ function (52). When assessing the net state of immunologic function, the conventional approach has been to monitor the immunosuppressive drug level and to record the cytopenia status. These are nonpathogen specific and can be generally useful, but, given individual variation in pharmacokinetics and pharmacodynamics, drug levels do not provide sufficient means to assess immune function (53). Other nonpathogen specific biomarkers of immune function are immunoglobulin levels (54), serum complement factors, peripheral blood lymphocyte subpopulations, soluble CD30, and intracellular concentrations of ATP in stimulated CD4 cells (52). These are associated with risk for bacterial infection and will not be discussed here, but certain biomarkers have been associated with risk for virus infection. For example, hypogammaglobulinemia (HGG) is not uncommon in the first year post transplant (55, 56), especially in those receiving mycophenolate mofetil (55), in those with bronchiolitis obliterans (57, 58), and in those receiving corticosteroid pulses after heart transplant (59). Although HGG is usually associated with bacterial infection (60), one meta-analysis (54) reported a nearly 3-fold higher risk of CMV during HGG. Clearly, transplant patients should be monitored for IgG levels, and IgG replacement therapy has shown improved outcome in certain groups of transplant recipients (61–63). With regard to complement activation biomarkers, C3 has not been associated with risk for virus infection. However, the lectin activation pathway for complement is influenced by mannose-binding lectin (MBL), and, in SOT recipients, MBL levels have been associated with an increased risk for CMV relapse post valganciclovir therapy (64). However, there is no clear consensus at this time on the need to monitor MBL levels.

Probably the best nonpathogen specific biomarker for infection is lymphopenia post SOT/HCT (16, 65). In general CD4+ lymphopenia is associated with CMV infection (66) and other infections. In the HCT recipient, lymphopenia can be related to GVHD, and it is likely that there are factors that influence the occurrence of both GVHD and CMV (67, 68). In liver allograft recipients, CD4+ cell counts $<300/\mu l$ place a patient at increased risk for CMV infection (52), and, in the HIV-1-infected SOT patient, a CD4+ lymphocyte count $<200/\mu l$ is associated with severe risk of infection similar to that seen in the general HIV-1 population, prompting the use of prophylactic antibiotics (69).

Finally, there are two other laboratory assays for nonspecific measurements of immune function. The first is based on the circulating level of soluble CD30, a glycoprotein related to the tumor necrosis/nerve growth factor family (70). In SOT, high levels have been linked to risk for graft loss (71), although the sensitivity and specificity of the assay used in this way is modest (72). CD30 levels still have not been convincingly correlated to risk of infection after transplantation. The second is the *in vitro* measurement of iATP in CD4+ T cells after nonspecific stimulation with phytohemagglutinin, a commercially available assay called ImmuKnow (73). Studies have linked low iATP to risk for CMV infection post lung transplant (74), EBV infection, and BK virus infection (75). At present, however, there is no recommendation on how best to use the iATP assay because its consistent performance is complicated by lack of sufficient experience in prospectively observed studies and by the vagaries of collection and processing time (52, 76). At this time, no recommendation can be made for use of the assay in SOT and HCT patient management.

CMV-Specific Immune Monitoring

Since the CMV serostatus itself imparts the main risk for CMV infection (77), the question arises as to how best to monitor the patient for CMV-specific T-lymphocyte function or lack of such function (78). The ability of T cells to make interferon gamma (IFNg) correlates with protection from CMV, and this raises the obvious question as to whether the IFNg response to CMV can guide preventive or therapeutic strategies (39, 79–81). Five commercial tests have become available for 4 types of CMV-specific assays of immune function; these include a CMV-peptide inducible intracellular cytokine release assay, the MHC-tetramer stain that measures the binding of antigen-specific HLA complexes to the T cell, the QuantiFERON-CMV assay (Qiagen

Inc.), and the ELISpot assay (T-Track CMV, Lophius Biosciences GmbH, Regensburg, Germany) and T-SPOT.CMV (Oxford Immunotec Ltd, Oxford, UK, and Marlborough, MA, USA) (82). The QuantiFERON-CMV assay is based on the release of interferon-gamma as measured by ELISA after an overnight incubation of whole blood with CMV-specific peptides, and this test has been most studied post SOT/HCT. In one such study (83), approximately one- third of CMV seropositive recipients actually lack demonstrable CMV immunity when inducible INF-gamma production is measured, and these patients are at risk for post SOT-CMV infection (80). In a multicenter study that focused on high-risk D+/R- recipients after discontinuation of antiviral prophylaxis, those with a positive QuantiFERON-CMV result at time 0, 1 month, or 20 months post valganciclovir prophylaxis had less late-onset CMV disease than those with a negative result (84). Results with the QuantiFERON-CMV assay suggest that spontaneous clearance of viremia occurs in 92% of patients with a positive assay result (81). The assay has FDA approval to claim that it "may assist" the clinician's ability to predict risk of CMV infection and guide decision-making in regard to treatment. Finally, the T-Track CMV- and T-SPOT. CMV-ELIspot assays are both in clinical evaluation in the setting of transplantation. While both methods are very promising, their role as clinical tools for assisting patient management remains to be determined.

Clinical CMV Disease, Treatment, and Prevention

Disease Course

In SOT patients, CMV-associated complications have not changed since the early descriptions of fever, malaise, with or without neutropenia and thrombocytopenia, and with or without subsequent fungal and bacterial complications (13, 14). In both SOT and HCT, the most dreaded CMV-associated complication is interstitial pneumonia (CMV-IP), but enterocolitis and hepatitis are more frequently seen complications. CMV-IP presents with dyspnea and oxygen desaturation and can quickly progress to respiratory failure. The use of antiviral prophylaxis has markedly reduced serious CMV-IP, but this complication remains a late problem both in the allogeneic HCT recipient with chronic GVHD (14) and in the SOT recipient on long-term immunosuppression. In fact, late onset CMV disease occurs in approximately 18% of SOT recipients depending on D/R serostatus, and late CMV disease has been observed in as many as 34% of D+/R- SOT recipients when prophylaxis is stopped at 3 months (78). Late occurrence of disease is due to a failure of development of CMV-specific cellular immunity in the R-recipient during the months of anti-CMV prophylaxis (79). Of interest, delayed implementation of CMV prophylaxis by as few as 14 days post transplantation has been associated with less late-onset CMV disease (85). This suggests that providing some limited exposure to CMV is important for reconstitution of CMV immunity after SOT is consistent with the known control of CMV in R+ SOT recipients with CMV-specific cellular immunity (86).

CMV disease can present in a variety of ways, and several serious syndromes are associated with the specific transplant patient groups. For example, with heart and lung transplantation, bronchiolitis obliterans can occur following CMV infection and following acute rejection. Bronchiolitis obliterans is defined as a decline in forced expiratory volume in one second, $<80\%$ posttransplantation baseline, or histological presence of obliterative brochiolitis (87). In both HCT and SOT, CMV infection can be associated with significant indirect effects, including poor graft function with acute and chronic rejection (88), increased bacterial and fungal infection (13), and graft loss (89), as well as increased mortality (17, 90). Acute rejection is associated with CMV infection in all solid organ settings (88), e.g., for heart transplant (91), for lung transplant (92), for kidney transplant (49, 93), and for liver transplant (94).

Prevention of CMV in the Seronegative Transplant Recipient

For the CMV-seronegative transplant recipient, for whom there is a CMV-seronegative organ donor, the risk of infection is determined by the exposure to posttransplantation blood product support. Except for the chance of community- or sexually acquired CMV infection, virtually all primary CMV infections can be prevented by careful preparation or processing of the blood components (95). For the CMV-seronegative HCT recipient of a graft from a CMV-positive donor, the risk of infection is a function of the number of cells in the graft (96). Overall, in HCT, virtually 90% of all CMV disease occurs in the CMV-seropositive recipient. In SOT, however, the option to use CMV-seronegative organ donors is not usually available, and the CMV-seronegative recipient of an organ from a CMV-seropositive donor will get a CMV infection in 80 to 100% of cases, and disease can occur in 50 to 70% if not given antiviral prophylaxis (13, 22) (Table 2).

CMV Disease Prevention: Antiviral Strategies

The approved antiviral agents for CMV prophylaxis following organ transplantation are IV ganciclovir, oral ganciclovir, oral valganciclovir (excluding liver SOT), and oral acyclovir (kidney SOT only). With ganciclovir (GCV), two strategies have been used; either all at-risk patients are treated for a defined period, or only transplant recipients with documented CMV infection are treated. The first approach is termed "general prophylaxis," and the second strategy is termed "preemptive" or early antiviral therapy. In either HCT or SOT, there is no best strategy for use of ganciclovir, in part because of its toxicity, and the clinical situation determines the appropriate approach. For example, the patient at high risk for CMV deserves prophylaxis, and the less risky patient is treated preemptively. In reality, hybrid strategies have developed in which prophylaxis is used during the highest period of risk, e.g., day 1 to 100 post SOT, and then preemptive therapy is used during periods of low risk (Table 2).

Regarding acyclovir in prevention programs, acyclovir was used with surprising success in modifying CMV infection and disease after HCT and SOT in the pre-ganciclovir era (97, 98). The mean 50% inhibitory dose of acyclovir for CMV strains is 63.1 ± 30.2 μM (99), and peak acyclovir levels in the plasma can range from 25 to 100 μM depending on the regimen. Valacyclovir, a prodrug of acyclovir, achieves blood levels comparable to IV acyclovir, and when given at a dose of 2 grams orally 4 times daily for >900 days after renal transplantation, significantly reduces the risks of CMV disease in high-risk D+R- patients (45% vs. 16%, $P<0.001$) and of acute rejection episodes (52% vs. 26%, $P<0.001$) (100). Similar inhibition of CMV reactivation has been reported in allogeneic HCT recipients (101). Despite this, neither acyclovir nor valacyclovir is approved for prophylaxis or treatment of CMV infection, and their use is only as an adjunctive agent in CMV prophylaxis regimens

TABLE 2 Prevention of CMV infection in transplant recipients

Transplant Organ	CMV Exposure	Risk Level	Preventative Regimen	Duration of Therapy	Comment
HCT -allo	D+ / R+ D - / R+	Intermediate to high	Preemptive	4 weeks	Continue preemptive therapy for high-risk patients[a]
HCT-auto	R+	Low	Preemptive	4 weeks	Monitor for CMV to D-60 for high risk patient
Kidney	D+ / R+	Low	Prophylactic	2–4 weeks GCV	Duration of therapy is based on immune status
	D - / R+	Intermediate	Prophylactic	3 months VGCV	
				2–4 weeks GCV	
				3–6 months VGCV	
Liver	D - / R+	Intermediate	Prophylactic	2–4 weeks GCV	Duration of therapy is based on immune status
	D+ / R-	High	Prophylactic	3–6 months GCV	
				2–4 weeks GCV	
				3–6 months GCV	
Heart	D - / R+	High	Prophylactic	2–4 weeks GCV	Duration of therapy is based on immune status
	D+ / R -	High	Prophylactic	6 months VGCV	
Lung	D - / R+	High	Prophylactic	2–4 weeks IVGCV	Consider using CMVIG[b]
	D+ / R-	High	Prophylactic	6 months VGCV	
Small Bowel	D - / R -	Low	Preemptive	—	Consider using CMVIG[b]
	D - / R+	High	Prophylactic	2–4 weeks GCV	
	D+ / R -	High	Prophylactic	6–12 months VGCV	
SOT not Small Bowel	D - / R -	Low	Preemptive	2–4 weeks GCV 6–12 months VGCV	Continue preemptive therapy for high-risk patients[a]

D = donor; R = recipient; + = CMV seropositive; - = CMV seronegative; GCV = ganciclovir; VGCV = valganciclovir; SOT = solid organ transplant; HCT = hematopoietic cell transplant; allo = allogeneic; auto = autologous.

[a]High risk = grade >2 GVHD; haploidentical donor; cord blood donor.

[b]Some experts recommend using CMVIG.

for select lower-risk populations for which ganciclovir or valganciclovir cannot be used.

Prevention of CMV after Solid Organ Transplantation

In SOT recipients, the management strategies are based on the variable risks for CMV based on organ type, D/R serostatus, and immunosuppressive regimen (Table 2). Unlike HCT in which rapid marrow recovery is the goal, in SOT there is less concern about the effects of marrow toxicity and prophylaxis with ganciclovir is the recommended approach. The advantages of prophylaxis are that it prevents CMV infection during the highest risk period of immunosuppression, decreases the indirect effects of CMV on the allograft, and removes the need for CMV monitoring. Prophylactic ganciclovir has been associated with both a reduction in organ rejection events and in coinfections (23, 102–104). A meta-analysis of 17 studies showed the clear superiority of prophylactic therapy over preemptive therapy for all SOT types (104). Prophylaxis versus preemptive therapy was associated with reduced rejection rates (26% vs. 53%) and reduced bacterial and fungal coinfections. The disadvantages are the side effects, including neutropenia, potential for the development of antiviral drug resistance, and prevention of CMV-specific cell-mediated immunity resulting in late-onset disease when the prophylaxis is stopped (23, 79). For this reason, prophylaxis does not prevent late-onset CMV disease, and approximately one-third of SOT recipients have been reported to get late-onset CMV infection post prophylaxis with D+/R- being at highest risk (105). When comparing prophylactic versus preemptive valganciclovir for kidney recipients, a double-blinded study showed increased CMV disease in the preemptively treated group (4.4% vs. 19.2%, P=0.003) (106). Hence, international guidelines recommend the use of a risk-based management plan in which those at highest risk, namely D+/R- recipients of SOT, receive prophylaxis; D+/R+ recipients at intermediate risk receive either prophylactic or preemptive therapy; and D-/R- recipients at lowest risk receive preemptive therapy (82).

The recommended duration of prophylaxis is 3 to 6 months for heart, liver, pancreas, and kidney SOT recipients and 6 months for lung and small bowel (Table 2) (80, 82, 104, 106–109). The kidney recipient is at lowest risk even in the D+/R- category presumably because of the lower tissue burden of CMV transferred to the recipient (110), and lung and small bowel SOT recipients are at the highest risk for CMV infection and require a longer period of prophylactic treatment. The current recommendations of the International Transplantation Society for CMV prevention in SOT are to begin with prophylaxis and then, based on the risk group, introduce preemptive therapy at the appropriate time (Table 2) (82). This type of approach has been shown to be effective in D+/R- liver transplant recipients, in which the strategy reduced CMV disease to approximately 10% at 2 years post SOT (111). During the preemptive therapy phase, weekly monitoring is done using a CMV PCR or antigen assay for 3 months and then spacing the monitoring according to need, based on concurrent immunosuppressive therapy. Quantitative PCR has been shown to be a more sensitive assay for detection of infection than the CMV antigen assay (94% vs. 24%) (89). The benefit of preemptive therapy is the reduction in side effects, less drug resistance, a reduction in cost, and, especially for the pediatric

patient, a reduction in the daily number of oral medications. However, this strategy creates a need to monitor, report, and act on results, and, in the real world of transplantation, this requirement in the critical posttransplantation period can be a burden. Several questions arise in the management of the SOT recipient:

- What is the correct dose of ganciclovir? Ganciclovir is given at a dose of 5 mg/kg IV twice daily. To lessen the potential marrow toxicity, the regimen has been converted to 5 mg/kg ganciclovir IV daily or 900 mg oral valganciclovir daily Monday through Friday.
- What is the optimal duration of prophylaxis? A large multicenter randomized study (100 days vs. 200 days of prophylaxis) demonstrated that the longer duration was significantly better in preventing CMV infection at 1 year post SOT [36.8% vs. 16.1%] with similar occurrence of adverse events (108). The longer duration of prophylaxis was associated with less CMV infection and minimal disease, but side effects of therapy prevented continued prophylaxis in some patients (105).
- Is there a use for oral ganciclovir? Oral ganciclovir is poorly absorbed, but its use has been approved in the U.S. for CMV prophylaxis after SOT based on its efficacy in control of CMV disease (22). In contrast, oral valganciclovir is well absorbed and can be substituted for intravenous drug during maintenance therapy for control of CMV reactivation. A large study comparing oral ganciclovir to valganciclovir in D+/R- organ recipients showed equivalence of the two drugs (112). However, valganciclovir is approved only for patients with AIDS or kidney, heart, and kidney-pancreas transplants. The dose of oral ganciclovir is 1 gram 3 times daily; the dose of valganciclovir is 900 mg once daily. Hence, valganciclovir is preferred for the convenience. The toxicity of these drugs is similar to the IV formulation of ganciclovir.
- How important is the duration of prophylactic therapy? Late CMV disease is a significant problem after the discontinuation of ganciclovir/valganciclovir prophylaxis. In a comparison of 100-day versus 200-day prophylaxis, the incidence of CMV disease was significantly less in the 200-day treatment group (37% vs. 23%) (108).
- Does use of preemptive therapy put a patient at significantly increased risk compared to the patient receiving prophylaxis? There have been no published randomized studies comparing these two strategies in nonrenal SOT. However, there have been comparative studies of preemptive versus prophylactic ganciclovir in renal SOT, with mixed outcomes. One such study in renal transplant recipients showed more CMV DNAemia in the preemptive group and more late CMV disease in the prophylactic group (113). A meta-analysis evaluating these strategies in SOT recipients concluded that both strategies for ganciclovir use, as well as prophylactic acyclovir, reduced CMV organ disease, but only prophylactic ganciclovir reduced the associated bacterial and fungal infection and death (104).
- Should valganciclovir be used in a liver transplant recipient? Valganciclovir is not approved for use in liver SOT recipients. Valganciclovir showed some inferiority to IV ganciclovir in this population due to an increased incidence of CMV disease in the valganciclovir group (112).
- What is the role for CMVIG? CMVIG is an immunoglobulin product derived from screening outdated plasma for elevated CMV antibody levels (114). A meta-analysis of randomized trials, comparing CMVIG with no treatment or with an antiviral, showed that CMVIG reduced the overall mortality and the CMV-related mortality and the incidence of CMV disease; however, it did not reduce the CMV infection or organ rejection (115). In heart transplant recipients treated with CMVIG, compared with those not treated or treated with antiviral only, the CMVIG group had significantly increased patient and organ survival at 7 years. Similar results have been observed in kidney and liver SOT recipients (13). Current guidelines recommend prophylaxis with either val-GCV or GCV, used orally or IV, with or without CMVIG for both heart and lung SOT recipients (82).

Prevention of CMV in the HCT Recipient

The prevention of CMV disease with intravenous ganciclovir was first shown in studies after marrow transplantation (116, 117). In HCT recipients, ganciclovir preemptive treatment has been shown to be associated with improved survival, whereas routine prophylaxis has no survival advantage (117, 118). The benefit of a preemptive strategy using ganciclovir was established in HCT when this marrow-toxic agent was given only to asymptomatic transplant recipients at the time of first laboratory evidence of active pulmonary CMV infection, a strategy that significantly reduced progression to subsequent CMV disease (116). Asymptomatic CMV pulmonary infection occurs after HCT (119), and approximately 60 to 70% of these patients will subsequently develop CMV-IP (116). Ganciclovir has also been used pretransplant (2.5 mg/kg IV every 8 hours on days -8 to -1 pretransplant and resumed at 6 mg/kg daily for 5 days per week at the time of engraftment) (120), with significant reduction in rate of CMV infection (20% vs. 55%) but with no significant reduction in CMV disease (10% vs. 24%). The problem is that prophylactic ganciclovir is associated with marrow toxicity and failure to develop CMV-specific immunity. Drug-related neutropenia occurs in approximately 30% of transplant recipients receiving ganciclovir (121). The median time of onset of neutropenia is 36 days (range 6–74 days) after starting treatment, and the neutropenia persists for a median of 12 days (range 4–20 days) (117, 118). Thus, strategies have been developed using short-course prophylactic GCV, followed by monitoring for CMV, and preemptive treatment when infection occurs.

As with prophylactic management, indicated in Table 2, ganciclovir is given at a dose of 5 mg/kg IV given twice daily for 7 days, and then maintained at 5 mg/kg once daily for 5 to 6 days per week for 2 to 6 weeks, based on the clearance of CMV DNA from blood. Thus, the use of preemptive ganciclovir is effective in preventing the morbidity of CMV infection, while sparing the toxicity of ganciclovir in those who are at lesser risk for disease. However, the need to identify those with significant CMV infection places a requirement for continued monitoring, and accurate monitoring is limited by the sensitivity of the assays used to detect CMV.

Several questions arise in management of the SOT/HCT recipient involving alternative antiviral agents:

- Foscarnet. The optimal method for prevention of CMV disease after HCT relies on a preemptive strategy using ganciclovir. At times, however, because of marrow toxicity, foscarnet should be used instead of ganciclovir. When the WBC falls to 1,000/μl, ganciclovir should be stopped and foscarnet begun. Also, during ganciclovir

treatment, it is not unusual to observe breakthrough CMV viremia, and this is usually associated with increased immunosuppressive therapy and not with drug-resistant virus (122). However, resistance to ganciclovir occurs, especially in SOT recipients (123), and, in this situation, foscarnet can be used alone or in combination with ganciclovir for CMV suppression (see discussion of Drug Resistance below). Although renal toxicity can be limiting, foscarnet can be safely used in the HCT population by following recommendations regarding pretreatment hydration (124).

- Cidofovir. Cidofovir has been used in transplant patients as second-line therapy for patients with CMV disease unresponsive to ganciclovir or foscarnet (125), but its renal toxicity severely limits its use.
- Letermovir. Letermovir is an orally available agent and has activity against both naturally occurring CMV and ganciclovir-resistant CMV (126). In a phase II placebo-controlled trial, the agent was shown to be very effective when used for prophylaxis in HCT patients (127). A phase II study using a preemptive strategy was evaluated in kidney transplant recipients and demonstrated a significant reduction in CMV DNAemia from baseline treatment (128). A phase III trial (NCT02137772) in HCT patients has completed enrollment and was in the analysis phase in 2016.
- Maribavir. Maribavir is an oral benzimidazole riboside, which blocks CMV DNA maturation by inhibition of UL97 protein kinase and has activity against strains resistant to CMV (129). In a study of 233 D+/R- liver SOT recipients randomized to receive either 1,000 mg ganciclovir orally three times daily or 100 mg maribavir orally twice daily, there was no difference in the incidence of CMV disease at 6 months post SOT, but when disease occurred, it occurred earlier in the maribavir group and more maribavir-treated patients had CMV DNAemia at 100 days and at 6 months (130). In addition, maribavir failed to meet endpoints in a phase III trial in HCT patients (131). It remains to be determined whether higher doses of maribavir would be effective.
- Brincidofovir. Brincidofovir, previously called CMX-001, is a lipid conjugate of the nucleotide analog cidofovir. In a placebo-controlled study in HCT recipients, the incidence of CMV events was significantly lower among patients treated with 100 mg twice weekly compared with placebo. This oral agent does have dose-limiting gastrointestinal toxicity, and diarrhea is a common adverse event in patients at doses of 200 mg weekly or higher (132). In a blinded study in 452 HCT recipients, 100 mg brincidofovir given twice weekly failed to meet the 24-week primary endpoint of prevention of CMV infection compared to placebo (Chimerix press release, December 28, 2015). Of note, there was less CMV infection through week 14 of treatment, but, in the 10-week post-treatment period, there was an increase in CMV infection compared to control. This was felt to be related to a higher use of immunosuppressive agents for treatment of presumed GVHD in the brincidofovir-treated group. Phase III trials in SOT were closed prematurely following the release of this information.
- Cyclopropavir. Cyclopropavir is a methylene cyclopropane analogue that inhibits CMV by UL97 kinase inhibition but with *in vitro* antiviral potency slightly greater than that of ganciclovir (133). This agent inhibits CMV by its effect on DNA synthesis, and, because resistance to cyclopropavir involves a different site on UL97, it is active against some ganciclovir-resistant strains of CMV (133). A phase I safety and pharmacokinetic study sponsored by the National Institute of Allergy and Infectious Diseases is being completed in normal volunteers (NCT02454699), and future clinical use of this agent will be based on these results.
- Leflunomide. Leflunomide, an inhibitor of protein kinase activity and pyrimidine synthesis, has broad antiviral activity, including *in vitro* activity, against CMV. It has immunosuppressive activity, is approved only for treatment of rheumatoid arthritis at a dose of 100 mg/day for 3 days (loading dose) and then 20 mg/day (maintenance dose) in patients without risk for hepatotoxicity (134). Leflunomide can be hepatotoxic and has produced liver failure and/or activation of hepatitis B virus in patients (135), and it can also cause myelosuppression. Although the drug does not reduce CMV DNA replication or protein synthesis *in vitro*, it does impair protein processing and viral assembly (136). There is a case report of clinical effect in drug resistant CMV (137), but there are no controlled studies to support antiviral effect (13). The largest published experience of leflunomide use for treatment of CMV in transplant patients was a single-center retrospective study of 17 patients who failed conventional treatment (137). In this report, there were a variety of CMV syndromes, and treatment with a loading dose of leflunomide at 100 mg/day for 3 days and then 20 mg/day maintenance dose for a median of 3.5 months was associated with clearance of CMV DNA from blood in 82% of cases, with a median time to clearance of the DNAemia of 1.9 months. A consensus of experts has recommended that caution be advised when leflunomide is used in SOT recipients for cases of severe CMV disease or in those with high viral loads (82). Similarly, in HCT recipients, there are mixed results with leflunomide and insufficient information from controlled trials to support its use (16).
- Artesunate. Artesunate has also been suggested as an alternative agent for drug-resistant CMV. This natural product is a derivative of artemisinin, the traditional Chinese medicinal used for malaria, and it has *in vitro* inhibitory activity against CMV (138). However, there is no convincing evidence of effect clinically (139), and there is no recommendation for its use in the setting of drug-resistant CMV.

Drug Resistance

Drug resistance to anti-CMV agents is most prevalent in the SOT populations and usually occurs as resistance to ganciclovir-related drugs. Resistance is seen in the HCT patient, but, perhaps because preemptive strategies are the more common approach in such patients, it is relatively rare in this population. The risk factors for emergence of drug resistance by CMV are D+/R- status, duration of ganciclovir/valganciclovir treatment, level of immunosuppression, and suboptimal drug dosing. In either SOT or HCT patients, when ganciclovir resistance occurs, it is usually after 6 weeks of therapy. CMV DNAemia often increases in the first 2 weeks post-ganciclovir preemptive treatment, and this is unlikely to be due to drug resistance (82). Drug resistance should be suspected if the patient has been on ganciclovir and CMV DNAemia persists. To confirm resistance, the CMV isolate is tested genotypically for mutations in either UL97 (CMV codons 400–670) or UL54 (codons 300–1,000). UL97 encodes the enzyme necessary for phosphorylating

GCV to its active form, and, because this mutation accounts for 90% of resistant strains, this assay is done first. UL54 encodes the viral DNA polymerase necessary for CMV DNA replication and is evaluated after the UL97 results, if necessary. The UL97 mutation prompts the switch to foscarnet. UL54 resistant CMV has more cross-resistance to both foscarnet and cidofovir, and thus is more problematic.

How does one treat a drug-resistant CMV infection? An international consensus group has developed an algorithm for managing a patient with ganciclovir-resistant CMV (82). For life-threatening or sight-threatening disease, the first action is to reduce the immunosuppression if possible. Then, foscarnet is added to the regimen while the patient's CMV isolate is sent to the laboratory for genotyping for likely drug resistance. For a major UL97 mutation, the switch to foscarnet is maintained. If there is no response in CMV DNAemia, mutations in UL54 are investigated and other antivirals are considered, but at this point there is no guidance from evidence-based medicine. Recommended approaches include increasing the ganciclovir dose to 10 mg/kg/dose, combining ganciclovir with foscarnet, adding CMVIG, or using other antivirals (82). If there is a UL97 mutation, cidofovir is likely to show cross-resistance and should not be used. Immunotherapy, using CMV-specific T cells for adoptive therapy, should be considered based on the patient status.

Cellular Immunotherapy for CMV

It was observed for many years that patients who were able to develop CMV-specific CTL function after allogeneic HCT survived serious CMV infection, and those who failed to develop a positive response to CMV infection often succumbed with CMV-IP (31). The posttransplant cellular immune response likely develops from memory cells present in the donor graft for patients receiving non–T cell depleted transplants (140). The possibility of augmenting donor- or recipient-derived viral immunity through adoptive immunotherapy has been a goal because the initial use of this method in a high-risk group of HCT recipients showed an absence of both CMV disease and CMV infection (141). Subsequent to this, several groups have demonstrated the feasibility of adoptive cellular therapy in this setting using various methods (142–145). Administration of CMV-, AdV-, and EBV-specific donor-derived T cells has been reported in small cohorts (146–148). It is even possible to derive CMV-specific T cells from CMV-seronegative donors (149) by induction of immunity *in vitro* or to use automated methods for cell product preparation outside of a GMP facility (150). The more recent ability to use third-party donors, sharing at least one HLA class I antigen, has shown promise in both SOT and in HCT recipients (151–154). The further ability to induce multivalent T-cell pools that recognize AdV, EBV, CMV, BKV, HHV6, and CMV greatly expands the potential for adoptive transfer as part of patient management (155, 156). In the near future, transplant physicians will be able to provide adoptive immunity to patients who have failed conventional antiviral therapies.

Vaccines for CMV

CMV induces neutralizing antibodies (NAb) during natural infection that prevent virus infection of either fibroblasts or epithelial/endothelial cells when studied *in vitro* (157, 158). NAb, targeting the gB, gM/gN, and gH/gL glycoproteins of CMV, block infection primarily of fibroblasts. The more potent NAb is one that is induced by a pentameric complex of gH//gL/UL128/UL130/UL131A and that inhibits epithelial/endothelial cell infection, protecting the human placental cytotrophoblast from infection (158). Induction of anti-gB antibody by vaccine in SOT recipients has been associated with shorter duration of CMV DNAemia and fewer days of ganciclovir treatment (159). An MVA-based vaccine encoding the pentameric complex has been developed and protects against infection in nonhuman primates (160). The evidence indicates that the vaccine can block endothelial cell infection (158), which suggests a role for the vaccine in protection not only of neonates but also of transplant recipients in the future.

For the transplant recipient undergoing graft-protective or GVHD-therapeutic immunosuppression, however, it is the loss of CMV-specific T-cell immunity that would need to be corrected by vaccination. For that reason, vaccines that target CMV proteins recognized by T cells are in the process of development. The first vaccine to complete a phase II trial in the transplant population was ASP0113 (TransVax, Vical/Astellis Pharmaceuticals Inc.), a DNA-based vaccine that encodes CMVpp65 and CMVgB proteins (161). In a placebo-controlled double-blind trial, the 80 donor-recipients had less CMV DNAemia, but not a significant reduction of CMV-specific antiviral treatments, than did the unpaired recipients. Based on these results, a phase III trial is now in place (NCT01877655) in the HCT setting. In addition, this vaccine is being evaluated in D+/R- kidney transplant recipients (NCT01974206) and in dialysis patients (NCT02103426).

A CMV peptide vaccine (CMV PepVax), in which a CD8-specific CMV pp65 peptide epitope is fused to a tetanus-derived helper cell epitope and used with a TLR9 agonist, is safe and immunogenic in volunteers (162). In a randomized nonplacebo controlled trial in HCT recipients (NCT01588015), CMV PepVax significantly decreased not only CMV DNAemia but also the use of anti-CMV therapy (163). Surprisingly, the vaccine group also had fewer leukemia/lymphoma relapses in the first year post HCT and an improved overall survival (163). Thus, there are encouraging data that suggest the potency of vaccines used in the SOT and HCT populations even during immunosuppressive therapy post transplantation. It is possible that, in the future, vaccines will become available for improved patient management.

EPSTEIN-BARR VIRUS INFECTION

Epidemiology, Risk Factors, and Pathogenesis of EBV Disease

In the general population, EBV infection is the cause of infectious mononucleosis (164, 165) and is associated with important neoplastic diseases, such as nasopharyngeal carcinoma (166), Burkitt's lymphoma (167, 168), non-Hodgkin's lymphoma in AIDS (169), and Hodgkin lymphoma (170) (see Chapter 25). In the transplant population, all aspects of these clinical manifestations of EBV infection can be seen, and, for this reason, EBV is one of the most important infectious problems after either SOT or HCT. Chronic immunosuppression, necessary to maintain the viability of the solid organ graft or the suppression of GVHD, permits either prolonged or more extensive EBV infection to occur, and it is in this setting that the various elements of EBV pathogenesis are seen.

From a historical perspective, it is important to appreciate the conceptual development, which explains the pathobiology of EBV-related diseases. The occurrence of

lymphomas in renal transplant recipients was first documented in 1969 (171), and a clear link to immunosuppression seemed apparent with the observation of a lymphoid malignancy at the actual site of injection of antilymphocyte globulin (172). Shortly thereafter, both the association of lymphoid malignancies with immunodeficiency and the interesting observation that the neoplasms associated with organ transplantation can regress with cessation of immunosuppressive therapy were made (173). The seminal observation that linked this clinical syndrome to EBV was the description of the X-linked lymphoproliferative syndrome in 1975 (see reviews [2, 174]). Subsequently, EBV was linked to malignant lymphoproliferation after infectious mononucleosis (175), organ transplantation (176, 177), and other immunodeficiency conditions (2).

The most significant feature of EBV infection in SOT recipients is the development of posttransplant lymphoproliferative disorder (PTLD). PTLD is an abnormal proliferation of lymphoid cells and can be heterogeneous in morphology, ranging from indolent polyclonal disease to aggressive proliferation of lymphocytes and plasma cells (2, 174, 177). PTLD was first reported in 1968 in two renal transplant recipients (178), and the prevalence of PTLD varies with the type of SOT and HCT. PTLD occurs rarely after T-cell-replete HCT, but the risk of PTLD is increased 30-fold by the use of T-cell depletion and 12-fold by the use of anti-T-cell therapy for graft versus host disease (179). In SOT, the incidence increases based on organ type, moving from low incidence in renal, to higher incidence in liver, then heart-lung, and then kidney/pancreas organ transplants (174, 180, 181).

Pathogenesis of EBV-Related Diseases after Transplantation

EBV infection usually begins with an initial lytic infection of the nasopharyngeal epithelial cells (182), and by this route, the virus gains access to the lymphoreticular system where latent infection of B lymphocytes and other cells occurs (2, 183). From early in the history of SOT, EBV infection was observed to be a major risk factor in PTLD in renal transplant recipients (184), and use of polymerase chain reaction methods have confirmed that quantitative assessment of EBV infection can identify those at greatest risk for PTLD (185). In healthy persons, an immune response controls the infection, but the total immunopathologic response to EBV infection, involving activated B, T, and NK cells, leads to enlarged lymph nodes, painful cervical lymphadenitis, tonsillitis, and splenomegaly, resulting in the clinical syndrome called "infectious mononucleosis." In the HCT/SOT recipient, EBV infection can occur in as many as 75% of patients (186), but in the absence of robust immunologic control on B-cell infection, progressive EBV infection occurs and results not only in the mononucleosis syndrome but also in B-cell lymphoproliferative disorders (187).

The EBV replication cycle involves two phases: a lytic phase in which proteins are made that lead to virus replication and cell death, and a latent phase in which the virus has an episomal existence, producing proteins that trigger tumor formation. Among the latent proteins, the latent membrane protein-1 (LMP-1) signals B cells for growth and differentiation, activating downstream pathways that lead to expression of anti-apoptotic cellular elements (188). Telomerase activity, which extends chromosomal telomeres and prevents cell senescence and apoptosis, depends on telomere-specific reverse transcriptase (TERT) and is crucial for most tumors. LMP-1 upregulates the expression of TERT, which further upregulates Notch2 and the transcription factor BATF, both important in B-cell function, and upregulation of gene expression causes an inhibition of an EBV lytic protein, BZLF-1, a major regulator of the EBV lytic cycle (189, 190). The overall effect is to force the EBV further into latency and extend the transformation process, potentially resulting in PTLD.

Similar to CMV, the source of the EBV in persons with PTLD appears to be the donor organ. In one case cluster, analysis of the EBV strains from a single donor and two recipients showed that the virus associated with the PTLD in both recipients was identical to that detected in the donor (191). However, using a DNA minisatellite probe to distinguish the DNA from lymphoblasts isolated from PTLD specimens, the B-cell lymphoid proliferation was recipient specific. With some exceptions, the cell of origin for PTLD is usually from the recipient after SOT, but in HCT PTLD it derives from the donor lymphoid cells, presumably because the recipient marrow is ablated (191, 192). The exceptions to this rule are noteworthy because of their importance to our understanding of the full spectrum of pathogenesis. In this regard, some studies have shown both recipient and donor cells in PTLD post marrow transplant (193) and PTLD of donor-cell origin after renal transplantation (194).

Pathology of PTLD

PTLD lesions are usually derived from B cells, with some occurrence of T-cell lymphoma (195), and the lesions contain B cells in all stages of differentiation with clonal, oligoclonal, or polyclonal characteristics. Clonal disease refers to the presence of one type of cell or EBV strain in a specimen. Oligoclonal disease refers to PTLD with more than one cell or virus characteristic, and polyclonal disease means that the PTLD specimen(s) contain many markers for either B cells or EBV (2, 196). For a description of the histopathology of PTLD, please search the University of Pittsburgh Transplantation Pathology Internet Services (http://tpis.upmc.com/changeBody.cfm?url=/tpis/PTLD/PTLDOver.jsp).

Risk Factors for PTLD

The risk factors for PTLD include type of transplant, age of recipient, and type and duration of immunosuppression (197–199). The incidence of PTLD is highest for intestinal and multivisceral transplantation, followed by lung and heart transplants, and the lowest incidence occurs in kidney and lung transplants (200). However, since more than three-fourths of all SOT involve kidney and liver transplants, most PTLD is seen in these patient groups. The most recent report on the incidence of PTLD in the time period 2010 through 2015 indicates a range from 0.5 to 2.9% in renal transplants and 0.8 to 3.6% in liver recipients (201). Ninety percent of these PTLD cases occurred within the first year after transplantation, and most, but not all, were EBV positive (201).

Based on the pathogenetic process outlined here, certain well-recognized risk factors determine which patients are more likely to develop PTLD. The development of EBV infection, especially primary infection, is the most important factor (202), and the amount of detectable EBV in the blood is directly related to occurrence of PTLD (186). Sequential analysis of EBV-DNA levels in peripheral blood leukocytes, from subjects with PTLD compared to SOT controls without PTLD, have shown that EBV-DNA levels increase in both groups with the induction of immunosuppression, but markedly elevated levels of EBV DNA are seen in the majority of patients before or at the onset of PTLD (203). PCR assays for EBV DNA in blood can detect elevated levels of

EBV DNA up to 3 weeks prior to onset of PTLD, and prospective use of quantitative PCR assays for EBV DNA can be used for early detection of PTLD (204). Of the immunosuppressive regimens that influence the ability to control EBV infection, the antibodies, which directly target T lymphocytes, such as the polyclonal antilymphocyte globulins and OKT3, are generally accepted to be very important risk factors for PTLD (205). In addition, cyclosporin A (CsA) and tacrolimus (FK506), which are used prophylactically to suppress graft rejection or graft versus host disease, are also accepted as significant risk factors for PTLD (206). As noted, the type of organ transplanted will also affect the risk of PTLD, with heart/lung having the highest risk among SOT recipients and T-cell-replete HCT having the lowest (180, 181). However, in HCT, it has been observed that certain subgroups, such as those with T-cell-depleted transplants, especially with higher numbers of stem cells (207), and matched unrelated marrow recipients (208), have a higher incidence of PTLD than allogeneic-related transplant recipients.

Clinical Manifestations of EBV Infection after Transplantation

Mononucleosis-Like Syndrome

Similar to infection in immunocompetent persons, a mononucleosis syndrome can occur in association with EBV infection in transplant recipients (205, 206). Thus, the clinical triad of sore throat with exudative pharyngitis, fever, and lymphadenopathy appears and can include other signs and symptoms, such as malaise, headache, anorexia, myalgias, and hepatosplenomegaly. Central nervous system complications, including aseptic meningitis, encephalitis, and the Guillain-Barré syndrome, can occur. Unlike infection in immunologically normal persons, in which the T-lymphocyte response contributes the "mononuclear" element to the hematologic findings, in the transplant recipient individual, one usually does not expect to see a true hematologic mononucleosis. The symptom complex of pharyngitis, fever, and lymphadenopathy should suggest EBV infection in this population. The serologic findings normally utilized for diagnostic purposes are not reliable in the immunosuppressed population, and direct EBV detection methods should be used for assessing infection (186, 196, 203).

Posttransplant Lymphoproliferative Disease

Although nonspecific signs of fever and malaise can be a hallmark of PTLD, especially persisting nonspecific signs and symptoms in the patient who has been treated for CMV infection, this disease ultimately presents as a focal or multifocal occurrence of lymphoid proliferation (206). The most common areas for disease are the central nervous system, the gut, and the allograft itself. The involvement of the allograft is particularly frequent in heart/lung recipients, where the differential diagnosis will include pneumonitis, graft rejection, and PTLD. Aggressive immunoblastic lymphoma can occasionally be seen, and this usually occurs in the first 100 days post transplant. For a complete review of this disease, see the online resource of University of Pittsburgh Transplantation Pathology Internet Services (http://tpis.upmc.com/changeBody.cfm?url=/tpis/PTLD/PTLDOver.jsp).

Hairy Leukoplakia

As in AIDS, chronic EBV infection of the transplant recipient can cause hairy leukoplakia (169). Oral hairy leukoplakia is a white or gray lesion on the tongue or oral mucosa due to epithelial hyperplasia. EBV and human papillomavirus have been associated with this syndrome. When EBV is present, it can be detected in the epithelial cells in these lesions, and it is known to replicate in linear form with high copy numbers of infectious virus. The lesion rarely undergoes malignant transformation.

Immune Monitoring for EBV Risk

As with CMV, demonstration of EBV DNA in the blood is a surrogate for inadequate immune control of virus replication (209, 210). As with monitoring for CMV immunity, EBV-specific immunity can be assessed using MHC-tetramer binding (211), T-cell intracellular cytokine release in response to EBV antigens (212), and ELISpot assays (213). These assays are not readily available to most clinicians, and therefore, at present, the monitoring of EBV DNA in blood is the best way to determine if the patient's T-cell immunity is functionally able to control EBV infection.

Treatment of EBV Infection

General Approach

PTLD is a life-threatening complication of transplantation and is associated with a mortality of more than 50%. Treatment involves a sequential combination of approaches, including reduced immunosuppression, use of rituximab with or without surgery, adoptive immunotherapy, and antiviral therapy. Reduction in immunosuppression can result in regression of PTLD, and therefore monitoring for EBV infection using PCR analysis and preemptive reduction in immunosuppression is the first aspect of patient management (214). The problem is 2-fold: graft rejection or GVHD can develop during such reduction in immunosuppression, and restoration of immunity can take longer than the progression of the PTLD. For gastrointestinal presentations of PTLD, local control of disease is often necessary, particularly if the disease associated with gastrointestinal bleeding, and, in general, surgical removal of the tumor at the site of bleeding. The role of antiviral therapy is unknown, but there are interesting anecdotes in which antiviral therapy appeared to improve PTLD. The treatment of PTLD was changed dramatically with the observations that adoptive humoral and cellular immunotherapy could positively affect disease progression (215). At present, treatment includes reduction of immunosuppression, surgical control of local disease, anti-B-cell therapy, introduction of donor T cells, and use of antiviral chemotherapy.

Antiviral Therapy

EBV is inhibited *in vitro* by several antiviral agents including acyclovir, ganciclovir, foscarnet, penciclovir, and interferon (216), as well as newer agents, such as maribavir and brincidofovir. However, except for oral hairy leukoplakia, in which acyclovir is effective therapy (217), there is little clinical benefit from antiviral agents during infectious mononucleosis (218), chronic mononucleosis (219), and even fulminant infection associated with X-linked immunoproliferative syndrome (220). The reason for the lack of clinical benefit is that antiviral agents are active only during the lytic phase of EBV infection and are not active during the latency phase. During lytic infection, EBV utilizes a virus-encoded DNA polymerase for DNA replication, yielding an extracellular infectious virion and causing cell death (221). During latent infection, EBV exists as a circularized, extrachromosomal, DNA plasmid (episome), and DNA replication is completed by means of cellular

polymerases (165). Because certain antiviral agents are inhibitors of viral DNA polymerase but not of cellular polymerase, antiviral agents are active in lytic, but not latent, infection (222). A primary example of a disease seen during lytic infection is hairy leukoplakia, which does respond to antiviral therapy (217). The latent form of EBV infection occurs primarily in B cells and results in activation and transformation of these cells into EBV-transformed, continuously replicating, lymphoblastoid cell lines. Thus, EBV-seropositive persons treated with acyclovir, years after the acute lytic infection, continue to have culturable EBV in circulating latently infected lymphocytes (223). For this reason, the effect of an antiviral on PTLD is usually less than desirable. Nevertheless, the outcome for PTLD is inversely related to the EBV-DNA levels in blood, and there are sufficient case reports associating antiviral use with patient improvement to suggest that a reduction in EBV DNA during the waning lytic phase may have an effect on the outcome of disease. Acyclovir, ganciclovir, and foscarnet in SOT have been associated with successful treatment in some patients (186, 224–227). Thus, although guidelines do not recommend the antiviral treatment in PTLD, it is rational to use antiviral agents early in the course of EBV infection in the immunosuppressed patient in an attempt to lower the EBV-DNAemia levels. Ganciclovir is more active than acyclovir against EBV *in vitro* and can effectively reduce the nasopharygeal excretion of EBV after transplantation (186). Prophylactic ganciclovir for CMV has been shown to reduce the incidence of PTLD by as much as 6-fold in SOT (228). For this reason, and because the patient usually needs anti-CMV coverage, ganciclovir is the agent of choice for treatment of the patient with rising EBV levels; treatment is provided as an adjunct to the reduction in immunosuppression.

In the future, antiviral agents may be used with TERT inhibitors for the treatment of PTLD. As noted, TERT is important in maintenance of tumor induction, and the inhibition of TERT can lead to expression of BZLF-1 and return of EBV to its lytic phase in EBV-positive tumor cells (189). Therefore, a proposed research strategy for treating PTLD would be to reactivate the EBV-lytic infection with a TERT inhibitor in an attempt to induce tumor cell death (189, 229–232). This would also promote immune recognition of EBV antigens and further enhance tumor killing. It has been shown that when ganciclovir is used with inducers of EBV-lytic infection, there is enhancement of cell death *in vitro* (233). It remains to be seen whether the combination of antiviral agents with inducers of the EBV-lytic phase will become effective therapies for PTLD.

Cellular Immunotherapy

Because remissions of both polyclonal and monoclonal tumors can occur after reduction or withdrawal of immunosuppressive therapy, adoptive cellular immunotherapy, directed toward improvement of immune function, has been attempted with remarkable results. Infusion of unirradiated donor leukocytes (approximately 1 x 10^6 CD3+ T cells/kg) into recipients of T-cell-depleted marrow transplantation, who developed PTLD, resulted in clinical responses within 8 to 21 days after infusion, including sustained remissions in 3 long-term survivors (215). Methods for preparing EBV-specific CTLs of donor origin have been developed, and these cells have been safely infused into both SOT and HCT recipients with PTLD (147, 153). Transfer of an EBV-specific T-cell receptor to produce a chimeric EBV-specific T cell has been shown to be feasible for treatment of EBV-associated malignancies (234). The more common approach is to induce panels of HLA-typed EBV-specific T cells and use these as third-party T cells for treatment of PTLD. In a phase II trial of transplant recipients with PTLD who failed conventional therapy, 33 patients were treated with EBV–specific T cells that were at least partially HLA matched to the recipient, with a 50% clinical response rate at 6 months (153). This type of approach illustrates the potential for cellular therapy to eliminate EBV complications after transplantation.

Humoral Immunotherapy for PTLD of B-Cell Origin

The availability of monoclonal antibody therapy for these B-cell lymphoid abnormalities has greatly improved the management of PTLD. An initial experience suggested that anti-CD21 and anti-CD24 antibodies could contribute to the control of oligoclonal B-cell PTLD (235, 236). Rituximab, a mouse-human chimeric monoclonal antibody with specificity for CD20, a B-cell surface antigen, has been approved for treatment of B-cell lymphomas (237), and this agent has become useful in both prevention and treatment of PTLD. Rituximab, at a dose of 375 mg/m^2 for 4 infusions over 1 month, resulted in an overall response rate of 69% in 32 PTLD patients and 20 complete responses. The initial approach to PTLD is reduction in immunosuppression, then rituximab therapy, and, for resistant disease, conventional lymphoma chemoradiotherapy (238, 239). Current recommendations suggest that rituximab should be used preemptively when EBV-PCR assays indicate increasing infection despite reduced immunosuppression (189).

HUMAN HERPES VIRUSES TYPE 6 AND TYPE 7

Epidemiology, Risk Factors, and Pathogenesis of CMV Disease

Epidemiology

For consideration in management of the SOT/HCT recipient, much more is known about HHV-6 than about HHV-7 (see Chapter 24). Consequently, the focus here will be on HHV-6, but differences in management of the two viruses will be noted. HHV-6 infection occurs early in life, and SOT/HCT donors and recipients typically have previously been infected with these agents. HHV-6 occurs as two variants: HHV-6A and HHV-6B, the latter accounting for most infections in children and reactivation in SOT/HCT recipients (240, 241). Following HCT, HHV-6 reactivates in 36 to 46% of recipients, and this occurs during the first 2 to 4 weeks post transplant (241–243). The exception is the pediatric transplant recipient who is HHV-6 negative and at risk for primary HHV-6 infection from the allograft (244). The incidence of HHV-7 infection is not as well documented (241).

Risk Factors

Specific risk factors for severe HHV-6 infection are not known, but the degree and duration of immunosuppression are undoubtedly important (245, 246). In addition, T-cell-depleting immunosuppressive regimens have been associated with virus infection (247). As with the other herpesviruses, the infection-naïve recipient of an allograft from an HHV-6 donor is at highest risk for disease (248).

Pathogenesis

The pathogenesis of disease during HHV-6 infection is not definitely described but is likely a function of progressive lytic infection in the patient with inadequate T-cell immunity.

Clinical CMV Disease, Treatment, and Prevention

Clinical Disease

Despite the relative frequency of HHV-6 infection post transplant (241), overt clinical disease, due solely to HHV-6, is estimated to occur in no more than 1% of transplant patients (249, 250). Clinical syndromes associated with HHV-6 infection include febrile dermatosis (251), encephalitis (252), gastroenteritis/colitis (253, 254), and hepatitis (255). In healthy children, HHV-6 is known to be neurotropic and is associated with febrile seizures, and an evaluation of encephalitis after allogeneic HCT has suggested a correlation with HHV-6 infection (243, 256). Prospective neurocognitive patient assessment and monitoring for HHV-6 infection in HCT recipients have associated HHV-6 infection with periods of delirium (243). In a separate study, HHV-6 DNA was observed in cerebrospinal fluid (CSF) specimens from HCT recipients with CNS symptoms in as many as one-quarter of cases (257). HHV-6 encephalitis is associated with seizures and an abnormal EEG but not with CSF pleocytosis or pathognomonic findings from imaging studies. In addition, HHV-6 infection overlaps with CMV infection and has been associated with pneumonitis and with marrow failure in syndromes similar to CMV.

Diagnosis

The diagnosis of HHV-6 infection is based on direct detection of HHV-6 in blood, CSF, or tissues. The most reliable assays are the HHV-6 DNA PCR on noncellular body fluid or an RNA-based nucleic-acid detection assay (258). Testing of cellular material, such as peripheral-blood mononuclear cells (PBMC), runs the risk of detection of latent virus (241). In addition, approximately 1% of seropositive persons have HHV-6 DNA integrated into host genomic DNA, and if DNA PCR is performed on PBMC, the result can be misinterpreted as a high HHV-6 viral load (259).

Antiviral Treatment

HHV-6 is susceptible to ganciclovir and foscarnet and relatively less susceptible to acyclovir (260). The problem with treatment is deciding whether a clinical syndrome is due to HHV-6 and worthy of treatment. In general, HHV-6 detection is not a clear indication for treatment because most infections are asymptomatic and transient (261). Organ-specific syndromes should have specific pathogens excluded before concluding that HHV-6 is the causal agent. Ganciclovir, foscarnet, and cidofovir are active against HHV-6 (260), but these agents are not approved for treatment of HHV-6. Nevertheless, despite the absence of large published studies, treatment is recommended, particularly for HHV-6-associated encephalitis, using one of these agents, usually ganciclovir or foscarnet.

- Should HHV-6 infection be monitored and treated preemptively? HHV-6 infection occurs more frequently than does HHV-6 related disease, and, for this reason, it is not recommended currently to monitor and treat preemptively (241).
- Should ganciclovir or foscarnet be used for treatment of HHV-6? There has not been a head-to-head clinical comparison of these two agents in HHV-6 encephalitis; however, there are anecdotal reports of success with either ganciclovir or foscarnet (241). *In vitro*, HHV-6B is usually susceptible to both agents, and both HHV-6A and HHV-6B are resistant to acyclovir and penciclovir (260). Of note, among HHV-6A isolates, more are resistant to ganciclovir than to foscarnet, and HHV-7 is resistant to ganciclovir (260). The decision about which agent to use must be made on clinical grounds by the physician.
- Does preventive treatment for CMV with ganciclovir protect against HHV-6 disease? It is likely that ganciclovir has an effect on HHV-6 reactivation during preventative ganciclovir use, but there is no clear documentation of this. The median time for HHV-6 is infection 20 days in HCT recipients (243), prior to preemptive ganciclovir use. There is a rare case report of HHV-6 encephalitis that occurred during ganciclovir preemptive therapy (262). There is also an observation of HHV-6 encephalitis following discontinuation of foscarnet therapy (263).

HUMAN HERPES VIRUS TYPE 8

Epidemiology, Risk Factors, and Pathogenesis of CMV Disease

HHV-8 (see Chapter 26), like EBV, is associated with tumors in the transplant setting, and, like EBV, there are both neoplastic and nonneoplastic complications of HHV-8 infection (264). HHV-8, also known as Kaposi sarcoma herpesvirus (KSHV), was discovered in 1969 (265), and the first case in SOT occurred in 1994 (265). HHV-8 is a problem in HIV-infected recipients of SOT because of its association with male-to-male sexual transmission. The virus has also been transmitted in the D+/R- setting (seronegative recipients of HHV-8 positive allografts [266, 267]) with resultant disease (268). Donor-derived KS tissue itself can also be transmitted to the immunosuppressed D+/R- recipient (266). Unlike the SOT experience, the occurrence of PTKS is rarely seen in HCT recipients (269). In SOT, there is a rare occurrence post transplant of HHV-8-associated primary effusion lymphoma (PT-PEL) and multicentric Castleman's disease (PT-MCD) (see review 269).

Clinical HHV-8 Disease, Treatment, and Prevention

The incidence of posttransplant KS (PTKS) varies with the prevalence of HHV-8 seropositivity of the region (270). Like EBV PTLD, PTKS is most commonly seen in kidney and liver SOT because these are the most frequent allografts (271, 272). Disease occurs a median of 30 months post transplant with 70% of patients presenting with cutaneous or mucosal lesions and the rest, with visceral lesions (269). Nonneoplastic disease, associated with high virus load and poor outcome, can be seen in patients presenting with high fever, maculopapular rash, and sepsis-like signs and symptoms (269). Patients can have a plasmacytic lymphoproliferation, lymphadenopathy that mimics EBV PTLD, acute bone marrow failure, hepatosplenomegaly, and liver-enzyme elevation mimicking acute hepatitis, with or without hemophagocytic syndrome (HPS) (269).

Diagnosis

The diagnosis of HHV-8 associated PTKS or PTLD is made by HHV-8 DNA level in blood and tissue and by clinical pathologic review of tissue biopsy. However, the latter is most important in diagnosis since >50% of PTKS can be HHV-8 DNA negative in blood (269). But like EBV and CMV, in patients with elevated HHV-8 DNA, the virus load in blood can be monitored as a marker of disease.

Treatment

As with EBV PTLD, the initial treatment of PTKS is reduction in immunosuppression or change to regimens

containing sirolimus (273, 274). The effect of reduced immunosuppression in this disease can be very effective (264, 275). For selective lesions, especially if associated with bleeding, surgical excision or cautery (276) will be necessary.

Antiviral and Other Chemotherapy

Like EBV, antiviral agents are inhibitory during the lytic phase of infection; thus, ganciclovir, valganciclovir, foscarnet, and cidofovir are active against the virus *in vitro* (277, 278). Antiviral treatment of KS has met with mixed success with small studies indicating some good outcomes (279–281) and larger studies showing minimal effect on KS (282). A preemptive treatment study using ganciclovir in high-risk patients has reported a decrease in PTKS (283). As with EBV, it is possible that the role of the antiviral is to minimize the lytic phase of the infection and reduce the progression to a latent state of infection. In general, antivirals, such as ganciclovir, valganciclovir, or foscarnet, are indicated for high HHV-8 DNAemia (284). Direct intracavitary instillation of cidofovir has been used successfully for treatment of PT-PEL (285). It is not known which of the antivirals is most effective, but there is a report of foscarnet treatment for a severe primary HHV-8 infection with fever, pancytopenia, and hemophagocytic syndrome (286). In HIV-1 infected SOT recipients, it is important to control the HIV-1 viremia with appropriate antiretroviral therapy, and this alone can reduce HHV-8 viremia and produce an antitumor effect in KS (287, 288). For primary HHV-8 infection, there is a recommendation for cautious use of rituxan in HIV-1 infected SOT recipients (289, 290). For management of cancer chemotherapy for PTKS, PT-MCS, and PT-PEL, see a recent review (269).

OTHER HERPESVIRUSES

Herpes Simplex Virus and Varicella Zoster Virus

HSV presents clinically after transplantation with the same type of infection and severity as seen in other immunosuppressed populations, and these will not be reviewed here in detail. Infection usually derives from reactivation of latent virus, with HSV presenting as an oral or genital skin infection, with the potential for visceral infection in the gastrointestinal system or brain. VZV usually presents as herpes zoster with dissemination of infection in a proportion of patients depending on level of immunosuppression. HSV reactivation occurs in approximately 70% of HSV-seropositive transplant recipients and can be suppressed with acyclovir (250 mg/m^2 IV twice daily for 1 month after transplant). VZV reactivation occurs in as many as 50% of allogeneic transplant recipients, and this can be prevented by long-term use of acyclovir (800 mg orally twice daily), although this is not an approved indication (291, 292). For acyclovir-resistant HSV or VZV infection, foscarnet is substituted for treatment.

POLYOMAVIRUS INFECTIONS IN TRANSPLANT RECIPIENTS

Polyomaviruses are ubiquitous in humans, and asymptomatic infection is acquired at an early age (293). In the setting of immunosuppression, however, unusual syndromes can occur, the most common of which are due to BK virus, a cause of nephropathy (294), and to JC virus, a cause of progressive multifocal leukoencephalopathy (295). Excretion in the urine of both BK and JC virus is common in transplant populations (296). In addition, there are 11 other human polyomaviruses (297, 298) that can complicate post-transplant patient management, including TS virus found in the proliferative skin disorder trichodysplasia spinulosa (299), Merkel cell carcinoma virus (300), human polyomavirus 7–associated epithelial hyperplasia (301), and other human polyomaviruses (297).

BK Virus Infection

Epidemiology, Risk, and Pathogenesis of Disease

The polyomaviruses of humans are BK virus (BKV) and JC Virus (JCV), and these are reviewed in detail elsewhere in this volume (see chapter 28). BKV infection is ubiquitous in the population (302), and virus becomes latent in kidneys and urothelial tissue. It was isolated from the urine of a renal transplant patient in 1971 (294). In such patients, latent infection reactivates in approximately 15% in year 1 post transplant (SG), and progressive infection of renal tubules, glomeruli, and interstitium can lead to BKV nephropathy. Approximately one-third of such BKV reactivations will progress to BKV DNAemia, and 1 to 10% of patients with viremia will develop BKV nephropathy (see chapter 28 and review 303). In addition to renal allograft recipients, the disease is seen in HCT recipients and less frequently in other SOT recipients (304, 305).

Risk

High BK virus DNAemia is associated with nephropathy (306). Levels of BKV of 10^7 genome copies/ml in urine and 10^4 genome copies/ml in plasma are not unusual for patients with BK nephropathy (307). BKV DNAemia is more sensitive for prediction of BKV nephropathy (308). A variety of other risk factors leading to BKV reactivation have been reported relating to donor BKV serostatus and HLA mismatch with recipient, age, race, and presence of diabetes, but, except for immunosuppression, none have been substantiated (see review 303). As with other virus-related complications in SOT/HCT recipients, T-cell immunity is a key risk factor for disease, with BKV-specific CD8 T-cell responses serving to decrease the relative risk for disease (309–311).

Clinical Disease

BKV infection is silent in most infected transplant patients and presents either with declining renal function or hemorrhagic cystitis (312). BKV infection of the kidney produces an interstitial nephritis that mimics acute rejection, but it can be differentiated from rejection effects by the presence of viral antigen/DNA and by an increase in B-cell infiltrates (313). In addition to interstitial nephritis, BK virus is linked to ureteric stenosis and hemorrhagic cystitis.

Diagnosis

A classical sign of BK nephropathy is the presence of decoy cells containing viral inclusions in the urine (303); however, decoy cells and BK viruria are less sensitive than BK-virus DNAemia for positive predictive value for BK nephropathy (303). A kidney biopsy is recommended for patients with BKV DNAemia of $\geq 10^4$ GC/ml. The kidney biopsy is the definitive method of diagnosis. Biopsy will show viral cytopathology in the tubular epithelium, glomeruli, and collecting ducts, and interstitial inflammation and fibrosis, in which the pathognomonic sign is the presence of large T antigen of BKV on immunostaining of the tissue (314).

Treatment

Reduction in immunosuppression is the first approach to therapy. Screening with reduction of immunosuppression in BKV-infected patients has been shown to prevent allograft

loss due to BKV nephropathy (315, 316). It may be necessary to convert the immunosuppressive regime to an everolimus- or sirolimus-containing regimen (317, 318). In terms of antiviral agents, cidofovir and leflunomide have been used, but no survival benefit has been observed (319). In a randomized blinded, placebo-controlled prophylaxis trial, a 3-month course of levofloxacin, started at day 5 post renal allograft transplantation, was not able to prevent BKV viruria (320). Similarly, there are no controlled studies that support the use of cidofovir for BK virus infection. Nevertheless, although cidofovir is unapproved for this indication, single-center studies exist that support the use of low-dose cidofovir (1 mg/kg 3 times weekly) for treatment of symptomatic BKV infection (321). Brincidofovir has been used for BK virus nephropathy in case reports (322), but there is no evidence-based recommendation for its use at present. Other questions arise in management of this infection:

- When should SOT patients be screened for BKV viruria? Several studies have confirmed that BKV viruria occurs in approximately one-third of patients and peaks at approximately 3 months post transplant (323, 324), but, of the early infections, it is rare to see BKV nephropathy. But BKV DNAemia is more sensitive for disease prediction (308), and so the better screening test is the BKV-DNA PCR assay in plasma. For that reason, at centers with relatively low rates of BKV nephropathy, routine screening of blood can begin at 3 months post transplant and continue to 24 months (303, 315). However, for centers with higher rates of BKV infection, guidelines suggest starting at month 1 and continue with monthly plasma BKV-PCR DNA testing for 6 months until 24 months (325, 326).
- What BKV-specific immunologic monitors are available? As with CMV and EBV, demonstration of virus DNA in the blood or urine is a surrogate for inadequate immune control of virus (325). As with CMV-immunity monitoring, BK-virus T-cell immunity can be assessed using antigen-specific T-cell immunity (311, 327, 328), BK-specific T-cell immunity is associated with recovery of kidney graft function in recipients with biopsy-proven BK-nephropathy (311, 328). But until immunological assays are generally available, the monitoring of BK-virus DNA in blood or urine is the best way to determine if the patient's T-cell immunity is functionally able to control infection.

JC Virus Infection

Progressive multifocal leukoencephalopathy (PML) is a rare but serious occurrence in SOT and marrow transplantation (329). JC virus infects oligodendrocytes of the CNS leading to a fatal demyelinating disease characterized by hemiparesis, seizures, deteriorating mental status, and death. JC virus infection, although thought of as a CNS problem, has been observed in 37% of patients with interstitial nephritis after renal transplantation (296). At present, there is no specific treatment for this infection.

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Viral Heart Disease

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Viral pathogens are well known to cause injury, inflammation, tissue destruction, and remodeling of heart muscle. Indeed, viruses are among the most common inciting agents to cause a condition termed acute myocarditis. This condition may also be provoked by bacteria, other pathogens, as well as toxins and autoimmune diseases, each of which could mimic the appearance of viral myocarditis. The reason for this phenotypic mimicry is that myocarditis is a process characterized pathologically by an inflammatory infiltrate of the myocardium with death or degeneration of adjacent myocytes, not typical of the ischemic damage associated with atherosclerotic coronary artery disease. The inflammation and damage may involve myocytes, interstitium, vascular elements, and pericardium. The inflammatory process affects cardiac function adversely, causing either ventricular dysfunction, arrhythmias, or both. The acute process may persist and manifest as chronic low-grade tissue inflammation and fibrosis associated with cardiomyopathy and frank heart failure.

Many viruses can cause the same syndrome, and a particular virus can cause infections leading to a highly varied constellation of manifestations. Clinically, viral heart disease and acute myocarditis most commonly commence with a "flu like" picture, followed within days by symptoms and signs of congestive heart failure, including shortness of breath, exercise intolerance, and fatigue, and may be associated with abdominal pain, chest pain, palpitations, syncope, and sudden death. In infancy and childhood, viral myocarditis is usually a fulminant process with left ventricular (LV) or right ventricular (RV) systolic dysfunction with or without ventricular dilation, whereas adults may present less abruptly and mimic dilated cardiomyopathy (DCM), mainly with LV dilation and systolic dysfunction.

In recent years, the main advances in our thinking about viral syndromes are that the attack on heart muscle is part of a more holistic viral-immune-inflammatory-pathological-clinical systemic syndrome with multiorgan involvement, and that there is a temporal connection between acute viremic states and long-standing immunovirological perturbation and cardiomyopathy. Certainly, the contribution of inflammatory mediators in transient, acute cardiac dysfunction is also now recognized (1). Yet the challenge often arises that a thorough evaluation of the myocardial tissue in biopsies or at autopsy is not possible or inconclusive for accepted features of myocarditis.

ETIOLOGY

Most cases of community-acquired myocarditis in the North America and Western Europe result from viral infections (2–4). Early detection of viral infection relies on viral isolation and serology; however, these diagnostic approaches lack sensitivity and specificity. Molecular detection of viral genomes in heart tissue derived from biopsy, explants, or autopsy has enhanced these approaches. Although fraught with challenges of false-positives and the possibility of molecular detection in absence of commensurate myocardial pathology, PCR evaluation of cardiac samples from subjects suspected of having myocarditis has demonstrated a variety of viral genomes in human hearts.

In previous eras, mumps virus was found by molecular interrogation in association with endocardial fibroelastosis (EFE), a previously important cause of heart failure in children that has disappeared over the past 20 years (5). This form of cardiomyopathy was identified in children until the late 1960s, with an incidence of 1 in 5000 live births in the United States. Mumps virus genomic RNA sequences were found in 90% of myocardial samples from EFE patients analyzed (5, 6). Since that time, the incidence has declined significantly due to mumps immunization, and the status of mumps myocarditis in mumps-susceptible populations has not been recognized in recent years.

In the 1960s through the early 21st century, adenoviruses, especially serotypes 2 and 5 (7, 8), along with enteroviruses [coxsackievirus A (CVA) and B (CVB), echoviruses, and poliovirus], and particularly CVB (9–12), were the most commonly identified viral etiologies. Recently, parvovirus B19 has become the predominantly detected virus in patients with suspected myocarditis, especially in European countries (13, 14); however, a causal relationship with myocarditis has been more difficult to affirm (10,14–18). In Japan, hepatitis C virus (HCV) has been suggested to be a common etiologic agent of heart muscle disease, with the other viruses typically seen in North America and Europe playing a lesser role (19–21).

In addition to the frequently detected viruses mentioned above, other viral causes of myocarditis have also been

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reported, particularly in children, including influenza A and B viruses (22–25), cytomegalovirus (CMV) (26), herpes simplex virus (HSV) (27), rubella virus (28), varicella-zoster virus (29), Epstein-Barr virus (EBV) (30), human immunodeficiency virus (HIV) (31, 32), human herpesvirus 6 (HHV-6) (33, 34), dengue virus (35, 36), respiratory syncytial virus (RSV) (37), human metapneumovirus (38), Crimean-Congo hemorrhagic fever virus (39), parechoviruses (40–42), and chikungunya virus (43, 44). The acute stage of infection with chikungunya virus is characterized by fever, polyarthritis, and occasional rash and can be complicated by myocarditis and pericarditis (43, 44). Pericarditis is frequently a part of the phenotype of myocardial involvement by cardiotropic viruses, including classical clinical signs of friction rubs and pleuritic pain, and such membranous inflammation may become persistent (9).

The identified viral causes of myocarditis are summarized in Table 1.

EPIDEMIOLOGY

Myocarditis is a disorder that is clinically difficult to diagnose and thus underdiagnosed (2–4). In autopsy series, the prevalence of the usual lymphocyte-predominant form of myocarditis ranges from 4 to 5% from reports of young men dying of trauma (45) to as high as 16–21% in children dying suddenly. In adults with unexplained DCM, the proportion affected ranges from 3 to 63% (46, 47), although the large multicenter Myocarditis Treatment Trial, which was based on specific and strict diagnostic criteria (the so-called Dallas criteria; see below) reported a 9% prevalence (48). Hospital discharge data suggest an approximately 0.5–4.0% incidence of myocarditis as a percentage of prevalent heart failure (49). The thresholds for defining myocarditis are variable and depend on whether the diagnosis is derived clinically, serologically, pathologically, and/or molecularly. This variability contributes to the uncertainty surrounding the incidence of the condition (49).

Usually sporadic, viral myocarditis can also occur as an epidemic (45). Epidemics usually are seen in newborns, most commonly in association with CVB. Intrauterine myocarditis occurs during community epidemics as well as sporadically (50). Postnatal spread of coxsackievirus is via the fecal-oral or respiratory route (51, 52). The World Health Organization (WHO) reports that this ubiquitous family of viruses results in cardiovascular sequelae in less than 1% of infections, although this increases to 4% when CVB alone is considered (45). Other important viral causes, like adenoviruses (53, 54) and influenza A virus, are transmitted primarily via the respiratory route.

TABLE 1 Viral causes of myocarditis

Viruses
Adenoviruses
Arbovirus (chikungunya) virus
Arenavirus
Cardiovirus
Cytomegalovirus
Epstein-Barr virus
Hepatitis C viruses
Herpesviruses, especially human herpesvirus-6
Human immunodeficiency virus
Influenza A virus
Influenza B virus
Measles virus
Metapneumovirus
Mumps virus
Nairovirus
Parvovirus B19
Picornaviruses
Enteroviruses
Coxsackievirus type A
Coxsackievirus type B
Echoviruses
Parechovirus
Poliovirus
Rabies virus
Respiratory syncytial virus
Rubella virus
Vaccinia virus
Varicella-zoster virus
Variola virus

PATHOGENESIS

The process of myocardial and pericardial viral infection depends on viremic dissemination to target tissues following initial infection at the portal of entry. The portal (e.g., respiratory or gastrointestinal mucosa) affects the first and subsequent points of contact between the pathogen and the host's immune system (see Chapter 16). The elicitation of antigen-specific humoral and cellular immune responses in lymphoid tissues has a dominant influence on the pathophysiology of the viral infection, including the potential for immunopathologic responses within the heart.

Animal Model Studies

The immunopathogenesis of CVB and encephalomyocarditis virus has been studied quite extensively in murine models. CMV, HIV, and adenovirus models also have been described (55–62). In wild-type mice, CVB viremia occurs 24–72 hours after infection, and maximum tissue viral loads develop at 72–96 hours (12). Virus titers subsequently decline, with infectivity being rarely detectable beyond 14 days after inoculation, depending on the mouse strain and viral variant. Neutralizing antibody concentrations decline as virus titers increase, supporting a role for such antibodies in the viral clearance process. Along with T lymphocytes and natural killer (NK) cells, macrophages appear within 5–8 days after infection in the murine CVB model of myocarditis (12). Risk factors for severe myocarditis include age, mouse strain, viral variant, exercise, and gender (12). Pathogenetic mechanisms include direct viral myofiber destruction (63–65) and T-lymphocyte cytolysis (56–58 66, 67). Animals with absent or blocked T-cell function may have less evident myocardial injury, although the recognition that an extensive amount of damage is already done by viral mechanisms before immune cell responses occur is now widely appreciated. In most murine strains, the adolescent period is the one of most severe in viral heart disease. In BALB/c mice, great susceptibility also occurs between 16 and 18 weeks of age; males appear to have a more rapid and severe course of myocarditis than females. Estradiol has been shown to decrease severity, and testosterone increases immune-mediated

cytolytic activity in males. Either a preferential stimulation of T-helper cells or an inadequate stimulation of T-regulatory (cytolytic/suppressor) cells could explain why antibody responses to various antigens may be enhanced and cellular immune responses depressed in female murine models.

The NK cell population, especially the activating receptor NKG2D, is important in the pathogenesis of myocarditis (68). Animals depleted of NK cells prior to infection with coxsackievirus develop more severe myocarditis (56). NK cells are activated by interferon (IFN), an indirect and direct protective modulator of myocardial injury. Murine skin fibroblasts serve as target cells for CVB-sensitized cytotoxic T cells. The NK cells specifically limit the nonenveloped virus infection by killing the virally infected cells. Male mice are less efficient in activating NK cells. Presumably, the more efficiently viral clearance occurs, the less virally induced neoantigen production occurs, reducing recognition by cytotoxic T lymphocytes. T cells can effect injury by multiple mechanisms—causing accumulation of activated macrophages, helping with production of antibody, mediating antibody-dependent cell-mediated cytotoxicity, direct lysis by antibody and complement, and direct action of cytotoxic T cells (69).

In recent years, the roles of various matrix metalloproteinases (MMPs) in the pathogenesis of viral myocarditis have received attention (70–73). In essence, MMP-9 and MMP-12, through modulation of IFN-beta and -alpha, respectively, play a prominent role in the early inflammatory phase of myocarditis, complementing the roles of MMPs in later tissue remodeling and healing of injured myocardium (70–73).

Host genetic factors have been shown to affect the severity of disease, as well as the pathogenic mechanisms that participate in disease development (55, 60, 62, 74). Cytolytic T cells mediate a considerable amount of injury in myocarditis in BALB/c mice, with two distinct cytolytic T-cell populations being implicated—one recognizing virus-infected cells and producing direct myocytolysis and another that destroys uninfected myocytes and is believed to be an autoreactive lymphocyte. Complement depletion increases the amount of inflammation in this species, and no reactive immunoglobulin G (IgG) antibody is found in the myocytes. In DBA/2 mice, the T-helper cells indirectly mediate the course of disease, and complement depletion reduces inflammation. Cytolytic T cells are produced but apparently are not pathogenic; IgG antibody is found in the myocytes.

Human Observations

In humans, antibody-mediated cytolysis is found among 30% of patients with suspected myocarditis, as well as in almost all patients with proven infections with CVB or influenza A virus (67). A muscle-specific antimyolemmal antibody has been found in these patients and correlated with the degree of *in vitro*–induced cytolysis of rat cardiocytes. A CVB-specific cDNA hybridization probe detected virus nucleic acid sequences in patients diagnosed as having active or healed myocarditis or DCM (75, 76). Patients with unrelated disorders had no virus-specific sequences (75), suggesting that viral genomic material persists in patients with congestive cardiomyopathy or healing myocarditis for weeks or months. Although viral cultures are usually negative, continued viral replication may occur at a low level or abortively. The latter may conceal viral antigens by a process that prevents correct posttranslational processing of capsid proteins. Adult patients with myocarditis often have high neutralizing antibody responses to CVB1 to CVB6 (77). One hypothesis is that sequential infection and immune responses against several types of CVB are essential in the development of myocarditis; however, certain cases of myocarditis clearly involve exposure to only one type of CVB.

Defective cell-mediated immunity, compared with that in healthy controls, occurs in patients with myocarditis and DCM. The pathogenesis of adenoviral myocarditis differs from that of CVB (57, 66, 78), and the inflammatory infiltrate is substantially less in adenoviral infection (8, 79, 80), specifically the numbers of CD2, CD3, and CD45RO T lymphocytes seen in the adenovirus-infected patients as compared to those with myocarditis not due to adenovirus (80). Adenoviruses have a number of strategies for modulating the immune response that could affect the number of activated lymphocytes in the adenovirus-infected myocardium (57). Adenovirus E3 protein can protect cells from tumor necrosis factor (TNF)-mediated lysis, as well as downregulating major histocompatibility complex class I antigen expression. The early-region 1A (E1A) proteins can promote the induction of apoptosis (81) and inhibit interleukin 6 (IL-6) expression, as well as interfere with IL-6 signal transduction pathways. These functions of E1A may be pertinent to the development of the myocardial pathology seen in DCM. IL-6 promotes lymphocyte activation, which is reduced in adenovirus-infected patients. Apoptotic cells are also observed in the myocardium of patients with DCM.

Pathophysiologic Consequences

In the heart, viral infection triggers both interstitial inflammation and myocardial injury, resulting in loss of myocardial integrity, with consequent cardiac chamber enlargement and an increase in the ventricular end-diastolic volume (47, 52, 54, 82). Normally, an increase in volume results in an increased force of contraction, improved ejection fraction, and improved cardiac output as described by the Starling mechanism. However, in the setting of myocarditis, the myocardium is unable to respond to these stimuli and cardiac output is compromised. A series of interacting adverse changes occurs, reflecting the composite pathophysiological response of patients afflicted by myocarditis:

1. Interactions with the sympathetic nervous system may preserve systemic blood flow via vasoconstriction and elevated cardiac afterload. This sympathetic nervous system input results in tachycardia, a feeling of weakness, and diaphoresis.
2. Congestive heart failure ensues with disease progression. A progressive increase in ventricular end-diastolic volume and pressure results in increased left atrial pressure. This pressure elevation is transmitted retrograde to the pulmonary venous system, causing increasing hydrostatic forces that overcome the colloid osmotic pressure that normally prevents fluid transudation across capillary membranes. The associated symptoms include increasing shortness of breath, anxiety and even chest pain, and the consequence may be overt pulmonary edema.
3. Concomitantly, all cardiac chambers dilate depending on the extent of virus- and immune system–mediated injury, particularly when the LV is involved. This dilation, in addition to poor ventricular function, creates worsening pulmonary edema and worsening cardiac function. Ventricular dilation also results in stretching of the mitral annulus and resultant mitral regurgitation, further increasing left atrial volume and pressure.

4. During the healing stages of myocarditis, the fibroblast population expands and produces increased extracellular matrix, replacing previously normal myofibers and resulting in patchy interstitial and replacement scar formation. Reduced elasticity and ventricular performance can result in persistent heart failure. In addition, ventricular arrhythmias commonly accompany this fibrotic process as tissue inhomogeneity progresses.

PATHOLOGY

Gross Findings

Pathological findings are nonspecific in myocarditis, with similar gross and microscopic changes noted irrespective of the causative agent (47, 52, 77). Typically, the heart weight is increased and all four chambers are affected. The muscle is flabby and pale, with petechial hemorrhages often seen on the epicardial surface, especially in cases of CVB infection. A sero-sanguinous pericardial effusion may also be seen relating to the often-combined finding of pericarditis. The ventricular wall is frequently thin, although thickening related to edema may be found as well. The valves and endocardium are not usually involved. Mural thrombi may occur along the inflamed endocardium in the LV and RV, and small emboli are often found in the coronary and cerebral vessels (83). Coronary emboli, although rare, may produce areas of ischemia or injury with resultant cardiac arrhythmias that sometimes occur during the acute disease.

In cases of chronic myocarditis, the valves may be glistening white, suggesting that EFE may be the result of an *in utero* viral myocarditis (82).

Findings by Microscopy

An interstitial collection of mononuclear cells, including predominantly lymphocytes and phagocytic cells with occasional eosinophils (Fig. 1), is typical of early viral myocarditis (12). Polymorphonuclear cells are uncommon in acute viral heart disease. Viral particles have only rarely been documented, but molecular detection of viral genomes (discussed below) is readily possible during the first 10 days of infection. Extensive cell death can be seen on examination by light microscopy, a result of both necrotic and apoptotic processes in the myocardium (63, 84); evident effacement of cross striations in cardiac muscle fibers and accompanying edema is seen in severe infections, but especially with coxsackievirus. Perivascular accumulation of mononuclear cells has been described with CVB myocarditis, but infiltration by leukocytes is patchy and more aligned with sites of viral replication and injury than with the vasculature. In disease due to rickettsiae, varicella-zoster virus, and trypanosomes or other parasites, and in reactions to sulfonamides, vasculocentric lesions are a much more prominent finding (85–90).

Diphtheria myocarditis is frequently complicated by arrhythmias and complete atrioventricular block (91). The diphtheria exotoxin attaches to conductive tissue and interferes with protein synthesis by inhibiting a translocating enzyme in the delivery of amino acids (92). Triglyceride accumulates, producing fatty changes of the myofibers.

Bacterial myocarditis produces microabscesses and patchy focal suppurative changes. A combined perimyocarditis is also encountered frequently. Parasitic myocarditis caused by *Trichinella* has a focal infiltrate with lymphocytes and eosinophils, but larvae are usually not identified (85).

A severe myocarditis caused by *Trypanosoma cruzi* (88, 89) results in Chagas' disease. Rare in North America, Chagas' disease is endemic in South America, affecting up to 50% of some populations. Examination by microscopy reveals the organism as well as neutrophils, lymphocytes, macrophages, and eosinophils.

Sudden death in infancy may result from myocardial inflammation. James (93) described a resorptive, degenerative process in the His bundle and left margin of the atrioventricular node with the absence of inflammatory cells in cases he studied of infants who died in Northern Ireland. Further definition of the nature of involvement of the conduction system by viral infection has not been forthcoming since that early observation.

Giant cell myocarditis occurs with tuberculosis, syphilis, rheumatoid arthritis, rheumatic heart disease, sarcoidosis, and fungal or parasitic infections (94–100). Giant cells also occur in idiopathic (Fiedler's) myocarditis. There are two types of giant cells: cells originating from the myocardium and cells derived from interstitial histiocytes.

CLINICAL MANIFESTATIONS

Presentation of viral heart disease depends on the age of the affected individual, immune status, specific viral trigger, genetic factors, and the environment (10, 52, 77). Nonspecific influenza-like illness or episodes of gastroenteritis, respiratory illness, or rash may precede symptoms of congestive heart failure.

Newborns and Infants

Newborns or infants present with poor appetite, fever, irritability or listlessness, periodic episodes of pallor, and diaphoresis. Sudden death may occur in this subgroup of children (10, 52, 101). On physical examination, pallor and mild or moderate cyanosis in addition to classic symptoms of congestive heart failure are commonly noted. It is important to keep in mind that the younger the child, the more likely that the disease was triggered as an intrauterine event. While this form of myocarditis may be expressed as a chronic disease that mimics chronic DCM (5, 10), indeed the severity of acute illness is often profound and fatal. The earlier the infection, the more likely severe illness will be observed, reflecting the immaturity of the immune system and the comparative inability to fight a lytic viral infection. The prognosis of acute myocarditis in newborns is poor (102, 103). In one study, a 75% mortality rate was observed in 25 infants with suspected CVB myocarditis (102); most deaths occurred in the first week of the illness.

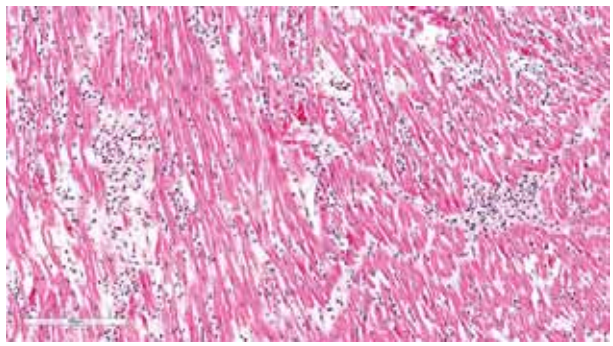

FIGURE 1 Endomyocardial biopsy specimen from a 19-year-old man with heart failure. Histopathology includes multifocal mononuclear cell infiltrates, areas of myocardial cell death, and apparent edema. Hematoxylin and eosin (H & E) stain; scale bar, 200 μm.

Children with myocarditis alone typically have symptoms for less than 2 weeks, whereas those with EFE have "viral" signs and symptoms for more than 4 months (Fig. 2). Mumps virus and CVB3 have been identified in the myocardium of infants with EFE (5), although a precise cause-and-effect relationship remains to be confirmed.

Children, Adolescents, and Adults

Older children, adolescents, and adults commonly have a recent history of nonspecific illness, typically with upper respiratory or gastrointestinal symptoms (with or without fever) 10–14 days prior to presentation (52). Initial symptoms may include lethargy, low-grade fever, and pallor. A child usually has decreased appetite and may complain of abdominal pain. Diaphoresis, palpitations, rashes, exercise intolerance, and general malaise are common signs and symptoms. Later in the course of illness, respiratory symptoms, such as breathlessness and cough, become more evident; syncope or sudden death may occur due to cardiac arrhythmias or arrest. Findings on physical examination are consistent with congestive heart failure (7, 52), as discussed above. Unlike with newborns, jugular venous distention and pulmonary rales may be observed, and resting tachycardia may be prominent. Arrhythmias, including atrial fibrillation, supraventricular tachycardia, or ventricular tachycardia, as well as atrioventricular block, may occur (52, 104).

Older infants and children have a better prognosis, with a mortality rate between 10 and 25% in clinically manifest cases. However, a subgroup of patients will present to urgent care centers in extremis from acute heart failure or arrhythmias, or die outside of care. Children may present with signs and symptoms of very common childhood disorders, such as a viral respiratory illness, gastroenteritis, or dehydration, and therefore are treated for these disorders initially. However, over hours or days, these children may rapidly deteriorate and succumb, usually after a cardiac or respiratory arrest. On autopsy, myocarditis is diagnosed. They may have associated hepatitis, pancreatitis, or encephalitis. These patients are extremely difficult to diagnose and, even if identified, have limited therapeutic options.

Complete recovery occurs in about 50% of patients (105, 106). Twenty-five percent of the patients continue to have an abnormal electrocardiogram or cardiomegaly on chest radiograph even though they were clinically asymptomatic. Abnormalities in the resting electrocardiogram may not be seen, but they may be brought out with exercise. Adult patients who recover may be asymptomatic at rest or with light exertion, but may demonstrate a reduced working capacity with exercise stress testing. As noted earlier, certain patients will have persistent pericardial inflammation as well (9).

DIAGNOSTIC EVALUATION

The diagnosis of myocarditis is often difficult to establish, but should be suspected in any patient who presents with unexplained congestive heart failure or ventricular tachycardia, especially in the absence of predisposing cardiac conditions. Appropriate diagnostic studies include the following (107).

Chest Radiography

Cardiomegaly with pulmonary edema is classically demonstrable on chest imaging.

Electrocardiography

Sinus tachycardia with low-voltage QRS complexes with or without low-voltage or inverted T waves are classically described. A pattern of myocardial infarction with wide Q waves and ST-segment changes also may be seen (108) (Fig. 3). Ventricular tachycardia, supraventricular tachycardia, atrial fibrillation, or atrioventricular block occurs in some patients (106, 109, 110).

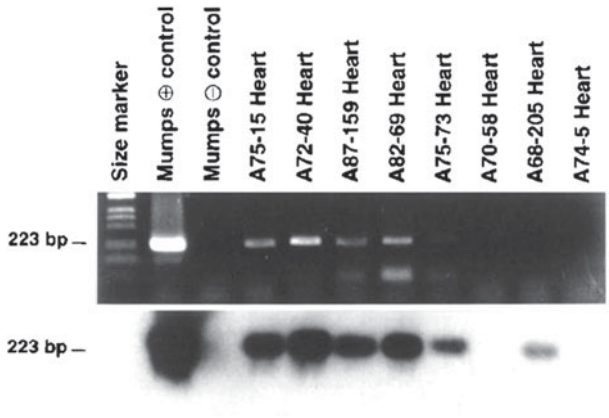

FIGURE 2 PCR analysis of fixed heart samples obtained from infants with endocardial fibroelastosis (EFE). Note the PCR-positive bands at 223 bp indicative of mumps virus. Sequence analysis confirmed the viral genome as that consistent with mumps.

Echocardiography, Ventriculography, and Magnetic Resonance Imaging

A dilated and dysfunctional LV consistent with DCM is seen on two-dimensional and M-mode echocardiography. Segmental wall motion abnormalities are relatively common, but global hypokinesis is the predominant finding. The regional dysfunction is at times shown to correspond with the areas of most intense myocarditis at various locations in the myocardium. Pericardial effusion frequently occurs. Doppler and color Doppler commonly demonstrate mitral regurgitation of a functional nature. Dilation of all cardiac chambers may be seen. Cardiac catheterization shows low cardiac output and elevated end-diastolic pressures. Cardiac magnetic resonance (CMR) imaging with gadolinium enhancement has emerged as a valuable diagnostic tool for myocarditis, providing evidence of locale and extent of inflammation in acute myocarditis (111–115).

Blood Tests

Indicators of inflammation, like white cell counts, C-reactive protein, and erythrocyte sedimentation rate, may be elevated in the blood during a myocarditis episode, but these are not in any way specific for myocarditis or subtypes caused by viruses. Given that myocardial injury does occur during acute myocarditis, it is not surprising that laboratory tests aimed at documenting such injury including markers such as creatine kinase MB isozyme (CK-MB), troponin I or T, or myosin light chains and others are typically elevated in the setting of myocardial injury (116–118). However, specific markers for acute viral myocarditis in routine blood studies are not available, and clinical context must be strongly considered in their interpretation.

Endomyocardial Biopsy

Right or left ventricular endomyocardial biopsy is used to examine pathological evidence of myocarditis, as well as for detection of viral pathogens. Pathologically, the features

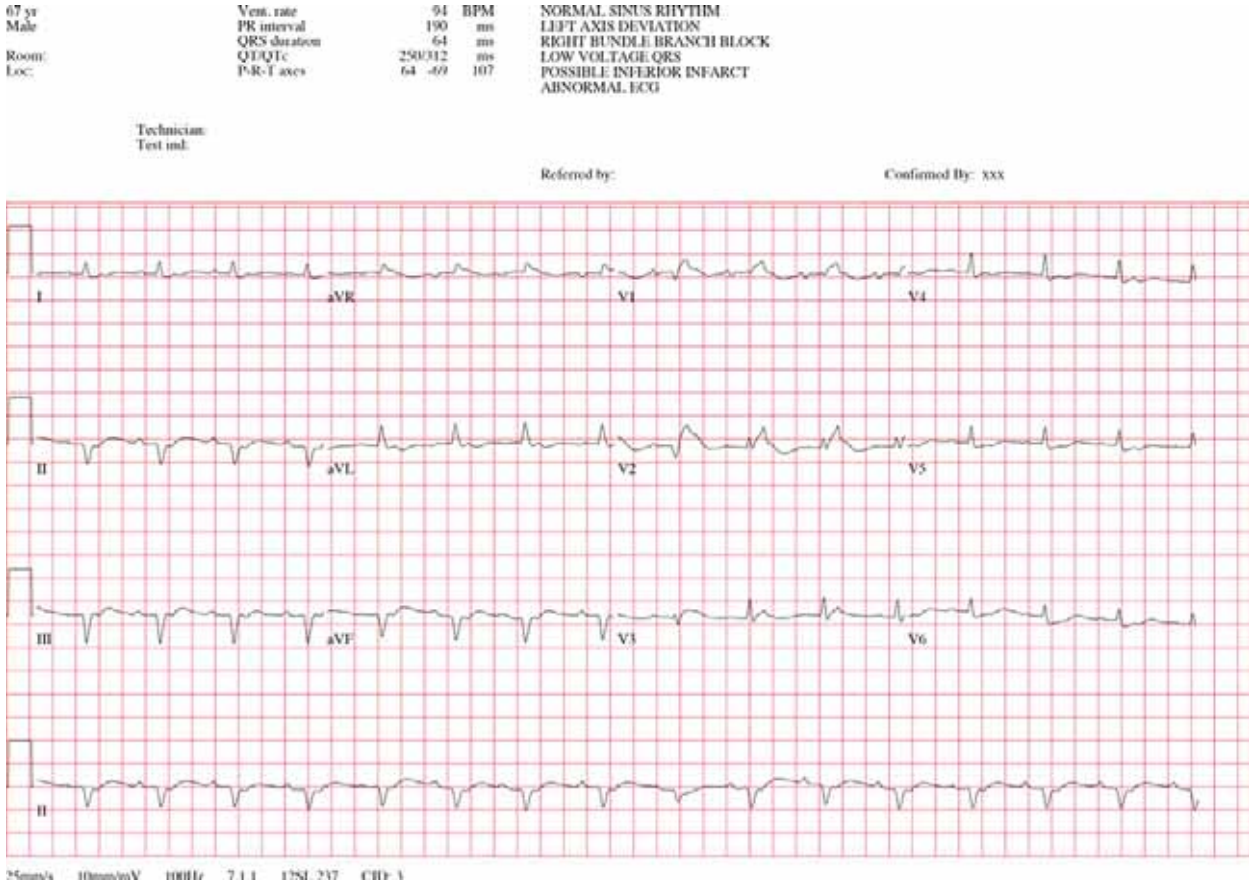

FIGURE 3 Electrocardiogram from a 67-year-old man with no prior history of heart disease, normal coronary arteries, and now severe acute heart failure and viral myocarditis. Sinus tachycardia and low-voltage QRS complexes with inverted myocardial injury pattern, particularly in the inferior ventricular wall, as well as left axis deviation and right bundle branch block are present.

include an inflammatory infiltrate and tissue damage (see Fig. 1), which is usually patchy and widely distributed in the ventricular myocardium. A mononuclear cell infiltrate inclusive of lymphocytes and macrophages, as visualized by immunohistochemistry, is always present in viral myocarditis and is required for the diagnosis of myocarditis (2). Myocardial biopsy has a widely variable diagnostic sensitivity, ranging from 3 to 63% of cases depending on the patient selection, biopsist capability, and expert level in pathological interpretation of findings (52,119–122). Because there are risks associated with biopsy, particularly in young children or those with severe ventricular dilation, certain centers have abandoned this procedure, particularly in young and small children (< 10 kg) and those with severe ventricular failure. For each patient, the purpose of obtaining a definitive tissue diagnosis by cardiac biopsy is balanced by the risks versus the ultimate benefit in clinical decision-making.

The Dallas Criteria

The Dallas criteria are based on histopathologic findings and define myocarditis as "a process characterized by an inflammatory infiltrate of the myocardium with necrosis and/or degeneration of adjacent myocytes not typical of ischemic damage" due to coronary artery or other disease (119). These criteria were evolved to standardize diagnosis for a major myocarditis treatment trial conducted in adult patients (106, 123). At the time of initial biopsy, a specimen may be classified as active myocarditis, borderline myocarditis, or no myocarditis, depending on whether an inflammatory infiltrate occurs in association with myocyte degeneration or necrosis (active) or only sparse infiltrate or no myocyte degeneration is evident (borderline) (48, 76). Repeat endomyocardial biopsy may be appropriate in cases where strong suspicion of myocarditis exists clinically; on repeat endomyocardial biopsy, histology may be classified as ongoing myocarditis, resolving myocarditis, or resolved myocarditis. The Dallas criteria more recently have been criticized as not reflecting the lower grade of inflammation that separates truly normal hearts from failing hearts with subtle immune cell infiltrates (124). Also, both active and passive cell death occur in the myocardium of patients and in model systems of myocarditis; thus, both apoptotic and necrotic features may be seen by microscopy (125–127).

Viral Studies

A positive viral culture from myocardium has been considered the diagnostic standard in the past. Viral culture of peripheral specimens, such as blood, stool, or urine, is commonly performed but is unreliable in identifying the causative infection. A 4-fold increase in virus-specific antibody titer correlates with recent infection (128, 129). However, these studies are nonspecific, because prior infection with the causative virus is commonplace, potentially yielding a greater rise in antibody titer than would be seen in a primary infection, and also because cross-reactive responses to related viruses may be observed.

First reported in 1986, *in situ* hybridization (ISH) was performed on myocardial tissue using molecular cDNA probes for coxsackievirus (Fig. 4) (75, 76) and more recently for parvovirus (Fig. 5A). While originally based on radioactive probes, ISH evolved to rely on nonradioactive probes and became more user friendly. Properly established under standard laboratory operating procedures, this method can detect as few as 50 copies of viral genomes in infected cells or tissues. The ISH technique, while very well described in experimental studies (63, 130), is highly specialized and not readily applied in most hospital settings, despite the fact that along with histopathological evaluation this technique provides evidence of active or resolving infections with enteroviruses and allows colocalization of injury, inflammation, and viral genomes (131, 132).

Detection and amplification of viral sequences by PCR from cardiac tissue samples is extremely sensitive and is typically specific (7, 8, 79). In 25–50% of cases, the enterovirus genome was initially identified by reverse transcriptase PCR (RT-PCR) (Fig. 5B, left panel) (8–10 16, 51, 79, 123, 133–135); however, no other viral genomes were sought in these early studies (133, 136, 137). Subsequently, PCR has been used to screen for other viral genomes within cardiac tissue specimens. Parvovirus (Fig. 5B, right panel) and adenoviruses (Fig. 6) were identified as commonly as enterovirus in heart tissue specimens of pediatric patients with myocarditis or DCM (Table 2) (7, 8, 10, 79). PCR analysis usually does not identify the viral genome in the peripheral blood of patients with myocarditis, but the viral genome can be identified in tracheal aspirates of intubated children with myocarditis (53). The great risk of PCR techniques is the common problem of sample or laboratory contamination and the possibility of finding genomes that are deemed a causative association with the clinical status, but in the absence of histopathological evidence of a myocarditic process. Similarly, viruses found by PCR in the respiratory tract do not secure a diagnosis of myocarditis. These realities temper one's belief in the molecular epidemiological profile of viral heart disease as currently known globally.

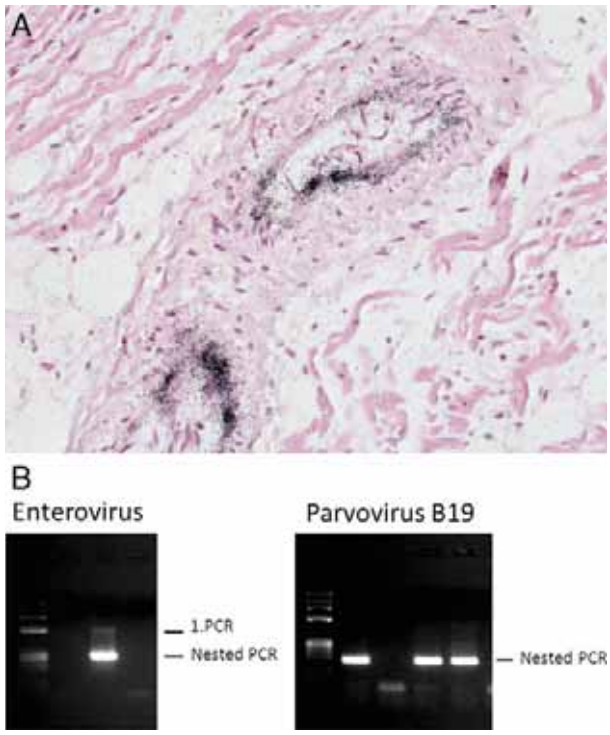

FIGURE 5 (A) *In situ* hybridization of acute parvovirus B19 infection of the heart is restricted to endothelial cells of small vessels as demonstrated by radioactive RNA/DNA hybridization. Cardiac inflammation is less prominent as compared to enteroviral myocarditis. Magnification, 400×. (B) PCR detection of enteroviral RNA (left panel) and parvovirus B19 DNA (right panel) by nested PCR in the myocardium of different patients with histologically proven myocarditis. Automatic DNA sequencing of viral amplification products confirms specificity of the PCR and allows the analysis of virus genotypes.

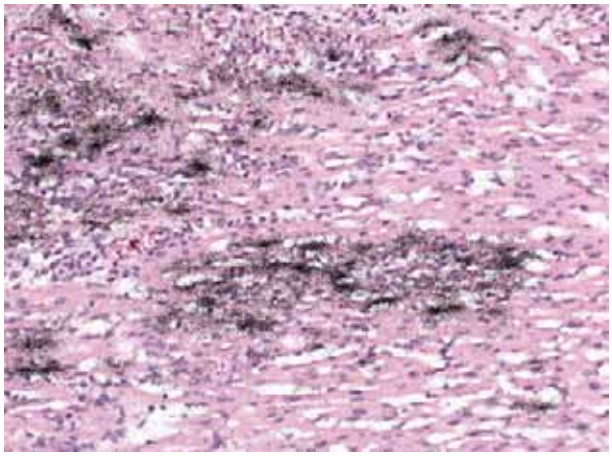

FIGURE 4 *In situ* hybridization for coxsackievirus B3 genome in myocardium of a newborn with lethal enteroviral myocarditis. Note replication of the virus in myocytes as visualized by radioactive RNA/RNA *in situ* hybridization (black dots) in close association with mononuclear inflammatory cells. Magnification, 400×.

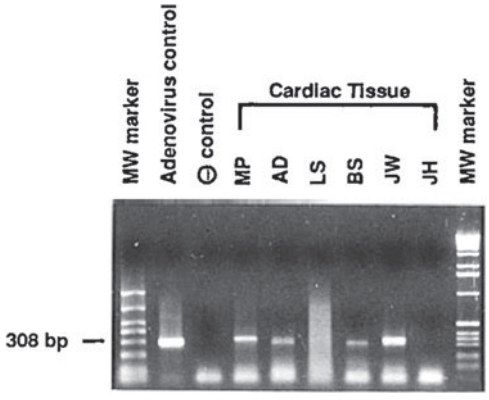

FIGURE 6 Nested PCR for the adenovirus genome. The agarose gel demonstrates a 308-bp PCR-positive band in the adenovirus-positive control lane, as well as in lanes designated MP, AD, BS, and JW, in which DNA was extracted from myocardial tissue samples obtained from patients with myocarditis. Patients designated LS and JH are PCR negative, as is the negative (–) control lane. MW, molecular weight.

TABLE 2 Viral etiologies of myocarditis in children by PCR analysis

Diagnosis	No. of samples	No. of PCR-positive samples	PCR amplimer (no.)
Myocarditis	624	239 (38%)	Adenovirus 142 (23%)
			Enterovirus 85 (14%)
			Cytomegalovirus 18 (3%)
			Parvovirus 6 (<1%)
			Influenza A5 virus (<1%)
			Herpes simplex virus 5 (<1%)
			Epstein-Barr virus 3 (<1%)
			Respiratory syncytial virus 1 (<1%)
DCM	149	30 (20%)	Adenovirus 18 (12%)
			Enterovirus 12 (8%)
Controls	215	3 (1.4%)	Enterovirus 1 (<1%)
			Cytomegalovirus 2 (<1%)

DCM, dilated cardiomyopathy.

DIFFERENTIAL DIAGNOSIS

Any cause of acute circulatory failure may mimic myocarditis. Other nonviral etiologies include other infectious agents such as rickettsiae, bacteria, protozoa and other parasites, fungi, and yeasts (83, 85,87–91138–142); various drugs, including antimicrobial medications (47), antipsychotics (143), and antitumor drugs (144); hypersensitivity, autoimmune, or collagen-vascular diseases (145–151), such as systemic lupus erythematosus, mixed connective tissue disease, rheumatic fever, rheumatoid arthritis, and scleroderma; toxic reactions to infectious agents (5, 30, 67, 152) (e.g., mumps or diphtheria); or other disorders such as Kawasaki disease and sarcoidosis (98, 153, 154). In most cases, however, the cause goes unrecognized or is poorly defined, and idiopathic myocarditis must be diagnosed (155).

LONG-TERM SEQUELAE

In patients in whom resolution of cardiac dysfunction does not occur, chronic DCM results, characterized by a dilated LV chamber, with or without LV diastolic dysfunction and/or right ventricular dilation and dysfunction (8, 51, 102,156–158). The underlying etiology of DCM is uncertain, but viral persistence and autoimmunity have been widely speculated. In addition, cytoskeletal protein disruption has also been demonstrated (159). Enteroviral protease 2A directly cleaves the cytoskeletal protein dystrophin, resulting in dysfunction of this protein (160–162). Because mutations in dystrophin are known to cause an inherited form of DCM (as well as the DCM associated with the neuromuscular diseases Duchenne muscular dystrophy and Becker muscular dystrophy), it is possible that this contributes to the chronic DCM seen in enteroviral myocarditis (163, 164). Adenoviruses also have enzymes that cleave membrane structural proteins or result in activation or inactivation of transcription factors, cytokines, or adhesion molecules to cause chronic DCM (57, 165, 166). Thus, it appears as if a complex interaction between the viral genome and the heart muscle tissues and cells occurs and determines the long-term outcome of affected patients.

As in mice, myocarditis in humans may have a genetic basis (167). Support for this tenet includes the frequent finding of myocardial lymphocytic infiltrates in patients with familial and sporadic DCM (168), as well as the few reports of families in which two or more related individuals have been diagnosed with myocarditis on endomyocardial biopsy. Of note, the shared receptor for four common viral causes of myocarditis (CVB3 and CVB4 and adenoviruses 2 and 5) is the human coxsackievirus and adenovirus receptor (CAR) (169, 170). The CAR plays an important role in embryonic development and the maintenance of normal cardiac function (64,171–173). Patients with DCM and young adults were shown to express increased levels of the CAR in the heart, suggesting a mechanism responsible for susceptibility to myocarditis (173). Genetic variants in the genome of this receptor might result in host differences leading to myocarditis, although this hypothesis requires study. The role of decay-accelerating factor (DAF), the coreceptor for CVB infections (174), also requires further exploration.

SUPPORT FOR VIRAL CAUSE-AND-EFFECT RELATIONSHIP WITH MYOCARDITIS

The increasingly common association of viral genomes within the myocardium in patients with myocarditis (80, 175, 176) is tempered by limited definitive data to prove that the virus causes ventricular disturbances directly leading to the clinical phenotype. Myocarditis has traditionally been defined as an inflammatory disorder, yet, even in this regard, definitive data to support the inflammatory concept are limited. In many cases of human myocarditis, frank, fulminant inflammation with lymphocytic or other infiltrate, edema, and cell necrosis with or without fibrosis is seen. But in other circumstances, little infiltrate, necrosis, or edema is seen in subjects with acute-onset heart failure and ventricular dysfunction. In both situations, the viral genome can be identified in up to 70% of those studied by PCR (80, 175, 176). In addition, PCR analysis of "control" specimens from subjects not thought to have clinical symptoms consistent with myocarditis and not having recent infectious or febrile illness very rarely detects a viral genome. However, because a relatively low percentage of biopsies are currently performed in children suspected of having myocarditis and, of those, a limited number have PCR performed on the myocardium, definitive cause-and-effect data are sparse. Occasional autopsy case materials provide definitive links between the enteroviruses and fatal heart muscle disease (177).

One human model system exists that has been used to study the relationship between the detection of viral genomes in the myocardium and ultimate heart failure. Cardiac transplant recipients undergo routine surveillance biopsies for rejection at most institutions, and in all cases, histopathological assessment is performed. At Texas Children's Hospital, all patients also undergo myocardial PCR analysis with screening for adenovirus, enteroviruses (including coxsackievirus), parvovirus B19, CMV, and EBV (178, 179). Detection of the viral genome in these heart biopsy samples has been shown to correlate with outcome. Heart transplant patients not having any PCR-positive studies during a 5-year follow-up period had a 96% 5-year survival rate, whereas those with at least a single PCR-positive result had a 5-year survival rate of 67% (163, 180, 181). Survival did not closely correlate with the level of inflammatory infiltrate seen on histopathology, and the specific virus identified in the myocardium appeared to be an

important variable regarding both the outcome and the inflammatory response. For instance, adenovirus has been shown to cause a lower level of inflammatory infiltrate than enteroviruses or parvovirus B19 (134, 181). Similar findings have been obtained for lung transplant recipients (182). How these posttransplant studies, complicated by the presence of an allo-immune response and by the presence of varied immunosuppressive agents, relate to the pretransplant connection between viral infection of heart muscle and patient outcomes is less clear. The experience of numerous laboratories with murine model studies has taught us the most thus far, with inference for the protean, difficult, and temporally vague human phenotype of myocarditis (70, 183, 184).

MANAGEMENT

Care of a patient presenting with a clinical picture and history strongly suggestive of myocarditis depends on the severity of myocardial involvement and the clinical status (2–4). Many patients present with relatively mild disease, with minimal or no respiratory compromise, and only mild signs of congestive heart failure. Such is the case because the most commonly recognized cardiotropic viruses are generally "high-attack, low-virulence" pathogens. These patients require close monitoring to assess whether the disease will progress to worsening heart failure and the need for intensive medical care. Experimental animal studies may suggest that bed rest may prevent an increase in intramyocardial viral replication in the acute stage (185–187). Thus, it appears to be prudent to place patients under this restriction at the time of diagnosis. Normal arterial blood oxygen levels should be maintained for any patient with compromised hemodynamics resulting in hypoxemia.

Management of Acute Heart Failure Related to Myocarditis

The current strategy for therapy in acute myocarditis includes hemodynamic support to achieve end-organ perfusion and urine output without "driving" the myocardium with inotropic agents. In the setting of hypotension, vasopressor infusions need to be used to maintain adequate blood pressure. The agents of choice include norepinephrine, epinephrine, and vasopressin. Inotropic agents such as dobutamine and dopamine may improve blood pressure and increase cardiac output but may have the associated cost of increasing heart rate and increasing mechanical stress on the heart, as well as increasing the possibility for arrhythmias. Phosphodiesterase inhibitors, such as intravenous milrinone, have been used to provide both inotropy and afterload reduction. However, their use may be limited by low blood pressure. Furthermore, the routine use of milrinone for acute heart failture (HF) was not associated with improved outcomes in the OPTIME-HF study (188). Digoxin therapy can also be instituted early, as it may provide positive inotropic effects while lowering the heart rate. If the patient is not requiring vasopressors, acute oral therapy with modulators of the renin-angiotensin-aldosterone systems (e.g., angiotensin-converting enzyme inhibitors) and sympathetic nervous system (e.g., beta-adrenergic blockers) can be started. When chronic oral therapy is necessary and hypotension is not present, an afterload-reducing drug, such as captopril, ramipril, or enalapril (189), may be used with beta-blockers, such as carvedilol or metoprolol. Diuretics therapy may be required to maintain a euvolemic state.

Although arrhythmias are commonly present in the acute setting, they should not be treated unless they are contributing to the symptoms or causing hemodynamic compromise (104). Sustained supraventricular tachyarrhythmias, such as atrial fibrillation, atrial flutter, or AV nodal re-entry tachycardia, may respond to digitalis or intravenous amiodarone. Sustained ventricular arrhythmias should be treated with cardioversion if associated with hypotension or hemodynamic compromise or an infusion of intravenous amiodarone if the patient is stable. Despite aggressive treatment of these arrhythmias, rapid deterioration to ventricular fibrillation, especially in the very young, may occur and should be treated immediately by direct-current cardioversion. The use of an implantable cardioverter defibrillator is rarely necessary in the acute phase. Chronic arrhythmias may persist long after the acute disease has passed (104). Thus, children who recover from myocarditis, regardless of etiology, should be monitored indefinitely. Complete atrioventricular block requires a temporary transvenous pacemaker because the patient may be dependent on a higher heart rate to generate an adequate cardiac output.

If the patient deteriorates despite maximal medical attempts at maintaining adequate tissue perfusion, then consultation for implantation of mechanical circulatory support should be considered (190–193). Some options include placement of a temporary ventricular assist device (VAD) like the TandemHeart (CardiacAssist, Inc., Pittsburgh, PA) or Impella (ABIOMED, Danvers, MA), or placing the patient on extracorporeal membrane oxygenation (ECMO) until the acute phase resolves. In the absence of resolution of shock, consideration for placement of a durable VAD such as Heartmate II (Thoratec Corporation, Pleasanton, CA) or HVAD (HeartWare, Framingham, MA) should be undertaken. In some circumstances, transplantation becomes necessary (163, 167), and outcomes may be best in patients who present most fulminantly (158). The number of such patients who definitely have a viral etiology is not established.

Immunomodulatory and Antiviral Therapies

Immunosuppresive agents have not been shown to improve outcomes in patients with acute viral myocarditis (106), but may improve outcomes in those with giant cell myocarditis (194). The use of immunosuppressive agents in suspected or proven viral myocarditis is controversial (195–197). Some animal studies have suggested an exacerbation of virus-induced cytotoxicity in the presence of immunosuppressive drugs, possibly due to reduced interferon production. The NIH-funded Myocarditis Treatment Trial analyzed the use of immunosuppressive therapy, including corticosteroids with either cyclosporine or azathioprine (106). Although the study was performed with adult patients, the results are potentially applicable to children. There was no difference in survival outcomes among patients treated with azathioprine and prednisone, cyclosporine and prednisone, or conventional supportive therapy (106). Immunosuppressive therapy was not beneficial in most patients with histologically confirmed myocarditis.

Another important therapeutic option is the use of intravenous gamma globulin in children with myocarditis. One nonrandomized clinical trial (74) used this agent in 21 of 46 children with myocarditis; patients who received this drug had better LV function at follow-up and a trend toward a higher survival rate at 1 year. Whether this approach proves to be beneficial or whether these early results mirror the early-published experience with corticosteroids remains to be seen (198, 199).

The efficacy of type-I IFN treatment in myocarditis has been studied with respect to viral clearance and prevention of progressive deterioration of LV function (136, 198, 200). These uncontrolled studies reported some effectiveness of IFN-alpha or -beta treatment in viral clearance and cardiac function improvement in patients with PCR-proven enteroviral or adenoviral myocarditis. Unfortunately, this impression was not confirmed in a multicenter clinical trial later on.

Potential therapeutics for enterovirus infections that involve the heart have been in progress for many years. Of note, pleconaril was developed as a steric inhibitor of picornaviral capsid protein binding to the CAR and other receptors (201, 202). While this investigational antiviral was widely used in the late 1990s and early 2000s on a compassionate care basis for treatment of acute, severe human myocarditis (203), it never received FDA regulatory approval. Vapendavir is a recently developed, more potent, and broader-spectrum capsid inhibitor; however, its efficacy in the treatment of myocarditis remains to be tested (204). Clearly, an antiviral strategy is especially pertinent to infants and young children whose lives literally depend on the balance between viral pathogenesis and host immune responses, and for which a drug like pleconaril or vapendavir may serve a pivotal role in tipping this deadly duel in favor of the host. Meanwhile, a vast amount of fundamental work on signaling mechanisms underlying enteroviral infections has been pursued with a view to identifying other targetable molecules.

There are also several available agents for influenza, adenovirus, and herepesviruses; however, clinical evidence of their value in the treatment of myocarditis is still lacking. For example, the phase III study of brincidofovir showed efficacy for adenovirus viremia in transplant patients, but this agent has not been studied for myocarditis (205).

Vaccination

Vaccination, except for influenza, is not currently available for the principal viral agents causing human myocarditis. The efficacy of the polio vaccine has led to the suggestion that a broadly reactive enteroviral vaccine, if possible, or at least a CVB-specific vaccine, could be beneficial for reducing the incidence of myocarditis or associated DCM. Early work indicates that immunization can be protective in mice (206). The possibility of success in this regard is supported by the success of the mumps vaccine, which all but eliminated this form of DCM.

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Viral Diseases of the Skin

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8

Viral infections cause a variety of cutaneous and mucosal manifestations that are either the result of primary viral replication within the epidermis or a secondary effect of viral replication elsewhere in the body. Three groups of viruses represent most primary epidermal viral replications: human papillomaviruses (HPV), herpesviruses, and poxviruses. Multiple virus families, including retroviruses, paramyxoviruses, togaviruses, parvoviruses, and picornaviruses, produce skin lesions secondarily. Other viruses, such as orthomyxoviruses and reoviruses, rarely induce skin lesions. Recognition of characteristic mucocutaneous manifestations of a variety of viral diseases is crucial. It either directly helps determine the etiologic agent or assists the clinician in deciding which additional diagnostic tests to order. Proper management of the patient can be initiated from the results of such tests.

CLINICAL MANIFESTATIONS

A wide spectrum of skin lesions can result from viral infections. For example, while infection with HPV is best known for causing verrucous papules, other manifestations of this viral infection include erythematous macules in epidermodysplasia verruciformis (EV), smooth papules in bowenoid papulosis, and fungating Buschke-Lowenstein tumors. Vesicles are considered the primary lesion in herpes simplex virus (HSV), varicella-zoster virus (VZV), and many coxsackievirus infections. However, the vesicles are often preceded by erythema and papules and followed by pustules, crusts, or shallow ulcers. Ulcers without other stages can be seen with cytomegalovirus (CMV) infections of the skin and mucous membranes as well as with HSV, VZV, or coxsackievirus infections of mucous membranes. Both macules and papules are seen with measles and rubella. Macules coalescing into larger erythematous patches are seen in Epstein-Barr virus (EBV), human herpesvirus 6 (HHV-6), and parvovirus B19 infections.

Some viruses induce skin changes that are highly suggestive of the diagnosis, such as the verrucous papules seen with papillomavirus infection or the smooth umbilicated papules resulting from poxvirus infection. However, other viruses produce nonspecific skin lesions, including urticaria, erythema multiforme, and petechiae. In these cases, a differential including viral and nonviral etiologies must be considered. Depending on the clinical picture, vesicles induced by HSV type 1 (HSV-1), HSV-2, or VZV may be diagnostic or may necessitate a broad differential diagnoses. Other herpesviruses, such as EBV, CMV, and HHV-6, produce skin manifestations less frequently and are most accurately diagnosed when the systemic manifestations of the viral infection are also considered. Cutaneous manifestations of viral diseases can range from very specific (e.g., dermatomal vesicles of herpes zoster) to very general (e.g., urticaria), and the differential diagnosis must take the patient's total clinical presentation into consideration.

PATHOPHYSIOLOGY

Viruses infect the skin via three different routes: direct inoculation, spread from an internal focus, and systemic infection. Viruses that infect the skin by direct inoculation include primary HSV, papillomaviruses, and most poxviruses (except smallpox). Primary VZV produces systemic infection with viremia and dissemination to the skin and mucous membranes. Recurrent VZV (shingles) or recurrent HSV reaches the skin from the sensory ganglia.

The effect of viral replication on infected cells may directly produce skin lesions, or the skin lesions may result from the host response to the virus. Alternatively, the lesions may be the result of the interaction between the viral replication and the host response. In general, viruses that replicate in the epidermis produce skin lesions directly. On the other hand, viruses that replicate elsewhere in the body typically produce skin manifestations via the host's response to viral replication. For example, the host's cell-mediated immune response to rubella and measles viruses is thought to be at least partly responsible for the skin manifestations associated with these viruses, and rashless measles can manifest as pneumonitis or central nervous system (CNS) disease in highly immunocompromised hosts.

DIAGNOSIS

Laboratory Diagnosis

Five general methods of laboratory diagnosis are available to confirm suspected viral diseases: viral culture, microscopic examination of infected tissue, detection of viral antigens, detection of viral DNA or RNA, and serology (see Chapter 15). Viral culture or polymerase chain reaction (PCR) are

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the preferred methods of diagnosis; both require adequate specimens. Viral culture is highly specific (>99%), but sensitivity depends on stage of lesion and proper collection technique and declines rapidly as lesions begin to heal (1). Sensitive virus isolation systems are not available for many viruses. If HSV-1 or HSV-2 is responsible for the lesion, a positive culture can be obtained within 1–2 days. Viral cultures are most likely to be positive if the sample is taken from the vesicular stage, whereas later stages of healing have lower yield. Positive cultures are more difficult to obtain from VZV, even when fresh vesicular fluid is used to inoculate the cell culture.

Evaluation of the involved skin by microscopy can reveal histologic changes consistent with a particular virus family, but is usually not helpful in identifying the specific virus responsible. For example, benign warts caused by different HPV types have a similar histologic appearance under the microscope. Histologic changes induced by HSV-1 and HSV-2, as well as by VZV, are similar to each other but distinctive from changes associated with other herpesviruses. A more rapid procedure in suspected HSV-1, HSV-2, and VZV infection is the Tzanck smear. Using fluid from an intact vesicle, the Tzanck smear is positive if acantholytic keratinocytes or multinucleated giant acantholytic keratinocytes are detected. Multinucleated giant cells are found in herpes simplex, varicella, and zoster. The Tzanck smear is insensitive (50%) and nonspecific. Another viral infection that can be diagnosed directly from smears from a skin lesion is molluscum contagiosum (MC). The presence of intracytoplasmic inclusion bodies will help to distinguish papules associated with MC virus from skin lesions of *Cryptococcus neoformans*, which can appear very similar in human immunodeficiency virus (HIV)-infected patients.

Among rapid diagnostic tests, perhaps the most frequently used for detection of viral infections of the skin is PCR and fluorescent antibody detection of HSV-1, HSV-2, and VZV. This technique distinguishes among these three viruses, in contrast to the Tzanck smear. Immunoperoxidase techniques are sometimes used to detect HPV capsid antigens; however, these techniques can lead to false-negative results in oncogenic types of HPV because the viral DNA may not have associated capsid antigens. Labor-intensive techniques such as electron microscopy or immunoelectron microscopy can be used to detect viral particles or viral antigens.

Assays to detect viral nucleic acid are becoming more widely used, especially when no effective culture or serologic assay is available. *In situ* hybridization allows not only detection of the viral nucleic acid but also histologic localization of the virus to specific cells. PCR primers can be designed to detect a range of viruses within a particular family (i.e., consensus primers) or may be specific for a particular virus (i.e., type-specific primers). Further information can be gained from *in situ* PCR, which combines the sensitivity of PCR with localization of the virus on histology. The application of PCR for diagnostic purposes is detailed in each chapter.

Differential Diagnosis

The differential diagnosis of various types of viral exanthemata requires the consideration of a spectrum of both viral and nonviral conditions. Vesicles may be due to HSV-1, HSV-2, VZV, poxviruses, hand-foot-and-mouth disease (HFMD) viruses, and other coxsackieviruses. Most vesicles develop into pustules during the process of healing. Therefore, the differential diagnosis of a vesiculopustular rash must include nonviral entities such as bullous impetigo, insect bite reactions, drug eruptions, contact dermatitis, gonococcemia, erythema multiforme, and sweet syndrome. Macules may be observed in rubella, EBV infection (infectious mononucleosis), and HHV-6 infection (roseola), as well as a variety of coxsackievirus A and B and echovirus infections. Nonviral etiologies of macules may include drug eruptions and bacterial infections (scarlet fever, Rocky Mountain spotted fever, and erysipelas). Macules may manifest with papules in measles, echovirus infections, and human parvovirus B19 infections (erythema infectiosum). Maculopapular lesions may also be seen in erythema multiforme, which is commonly of viral etiology (HSV) or may be associated with nonviral infections or drug eruptions.

Papules are seen in a variety of poxviruses and HPV infections, as well as in Gianotti-Crosti syndrome, which may be a manifestation of hepatitis B or another viral infection. Papules may also be seen with bacterial infection (*Bartonella* and *Mycobacterium*), fungal infections (*Cryptococcus*), and noninfectious conditions (seborrheic keratoses and basal cell carcinomas). Nodules may be observed in poxvirus infections (orf and milker's nodules), HPV (squamous cell carcinomas associated with HPV-16), or HHV-8 (Kaposi's sarcoma), as well as in mycobacterial and *Bartonella* infections (bacillary angiomatosis) and noninfectious tumors (basal cell carcinomas, squamous cell carcinoma, melanoma, and pyogenic granuloma).

Urticaria is usually associated with allergic reactions, including drug eruptions, but may be due to hepatitis B virus or coxsackievirus infections. Petechiae are seen in multiple viral infections, such as dengue fever and other hemorrhagic fevers (Lassa fever), but may occur in nonviral conditions producing thrombocytopenia. Ulcerations of the mucous membranes commonly occur with HSV-1, HSV-2, VZV, CMV, and HFMD viral infections. Anogenital ulcers in immunocompromised persons are sometimes due to CMV or may involve a coinfection of CMV and HSV. Oral ulcers of viral etiology must be distinguished from nonviral ulcers such as aphthous ulcers. Cutaneous ulcers may be related to stasis dermatitis or to other causes of decreased circulation.

LOCAL IMMUNITY TO VIRAL INFECTIONS

Not only does the epidermis serve as a primary line of defense against infections, but because of its anatomic structure, it also contains the basic elements needed for the immune response against infection. Therefore, the concept of skin-associated lymphoid tissue (SALT) has been proposed (2). SALT is made up of the following: (1) keratinocytes, which phagocytize foreign particles, release cytokines, and express major histocompatibility complex (MHC) class II antigens upon incubation with interferon-γ (IFN-γ); (2) epidermal Langerhans cells, which have surface expression of MHC class II, CD1, C3biR, and CD4 molecules and are the predominant scavenger antigen-presenting cell of the epidermis; (3) skin-trophic T cells, which in the epidermis include mainly "inactive" memory T cells of predominantly $CD8^+$ phenotype, although $CD4^+$ and $CD4^-$ $CD8^-$ gd^+ T cells are also present; and (4) skin endothelial cells, which direct cellular traffic in and out of the skin (**Fig. 1**).

DNA VIRUSES

The following sections highlight the mucocutaneous manifestations, diagnosis, and management of common viral infections. The reader should consult pathogen-specific

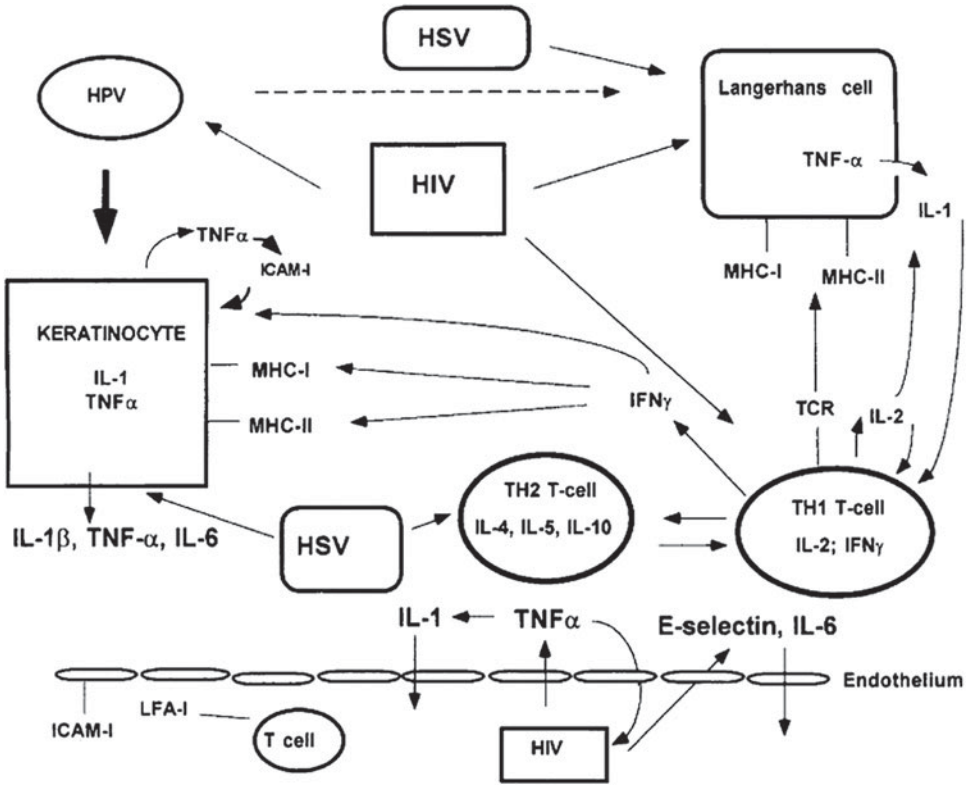

FIGURE 1 Pathways of the cutaneous immune response to infection with human papillomavirus (HPV), herpes simplex virus (HSV), and human immunodeficiency virus (HIV).

chapters for details on the virology and other features of individual viral agents.

Human Papillomaviruses

Epidemiology

Anogenital HPV infection is extremely common, with an annual incidence of 5.5 million cases in the United States (3, 4). Approximately 75% of sexually active adults will have had an HPV infection by age 50. Of these, approximately 60% have resolved infection, 14% have subclinical infection, and 1% have clinically evident lesions (5). Peak prevalence of anogenital HPV is in women younger than 25 years old; there is a second peak in women over age 55 (6).

Condyloma acuminata, or genital (venereal) warts, are the most frequently diagnosed sexually transmitted disease, with an annual incidence of approximately 1 million new cases in the United States (Fig. 2). Over 90% of cases of condyloma acuminatum are due to HPV-6 or HPV-11 and are clinically benign. Genital warts are most often spread by sexual contact, with a 60% transmission rate during sexual contact with an infected partner. The mean incubation period of HPV is 2–3 months but ranges from 3 weeks to beyond 8 months (7, 8).

HPV infection also appears to be very common in men but is less well studied. Most studies report prevalence in men comparable to that in women. However, men have lower seropositivity of HPV-6, -11, -16, and -18 (9).

The most significant risk factor for anogenital infection in both men and women is the number of sexual partners. HPV has also been positively correlated with smoking. A possible association with oral contraceptive use has been suggested (10). Male circumcision appears to reduce the prevalence of genital HPV in males (11), significantly reduces the prevalence and incidence of both low-risk and high-risk HPV infections, and increases clearance of high-risk HPV infections in their female partners. Male circumcision has been recommended as an effective intervention for reducing the prevalence and incidence of HPV infections in female partners (12). The mechanism by which circumcision helps protect against HPV infection is unclear but is thought to go beyond increased probability of good penile hygiene (13) and probably involves the reduction of HPV carriage in the penis (12). Furthermore, the keratinized stratified squamous epithelium of the penile shaft is likely

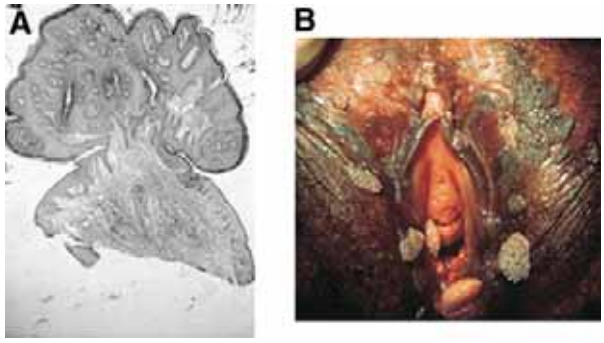

FIGURE 2 (a) Photomicrograph of condyloma acuminatum showing acanthosis, papillomatosis, and parakeratosis. (b) Condyloma acuminatum associated with human papillomavirus 6 (HPV-6).

less vulnerable to infection than the nonkeratinized mucosal lining of the prepuce (13).

Nongenital cutaneous HPV infections occur in 10% of children, with a peak incidence between the ages of 12 and 16 (14). Adults are also affected by cutaneous HPV but less commonly than children. Close personal contact is the main risk factor for transmission, as these lesions spread by direct skin-to-skin or skin-to-mucosa contact. However, transmission of both anogenital and nongenital cutaneous HPV infections depends on lesion location, HPV quantity in the lesion, type of contact, and immune status of the exposed individual.

Clinical Features

HPV infections can be categorized based on regional tropism—that is, whether they cause genitomucosal lesions, nongenital cutaneous lesions, or lesions associated with EV. The most prevalent clinical form of genitomucosal lesions is condyloma acuminata. These warts are exophytic, cauliflower-like lesions typically located near moist surfaces. Papular warts and flat lesions occur less commonly. These lesions are smaller and less obvious on exam than condyloma acuminata. Examination under a colposcope or other type of magnification may be necessary for identification (15). Anogenital HPV infection is commonly associated with cancer, as described in more detail below (see Chapter 28).

An uncommon manifestation of genital HPV infection is the Buschke-Lowenstein tumor, or "giant condyloma." This lesion typically manifests as a slow-growing, large, malodorous, cauliflower-like mass. These lesions resemble condyloma acuminata histologically, but exhibit both downward and upward growth, thus appearing locally invasive. Recurrences of Buschke-Lowenstein tumors are common (15).

Bowenoid papulosis is an anogenital neoplasia that manifests as multiple, verrucous, brown-red papules that may coalesce. Lesions are more common in females, in whom they occur around the labia minora and majora, inguinal folds, and perianal areas. In men, lesions occur on the glans or shaft of the penis (15).

Nonanogenital mucosal disease can occur in the nares, mouth, larynx, and conjunctiva. HPV from genital lesions can be transmitted to distant mucous membranes via orogenital sex (causing oral condyloma acuminatum) or nonsexually, as in cases of vertical transmission during vaginal delivery (16). In the latter case, HPV from vaginal warts may be transmitted to the oral or respiratory tract of the infant and manifest as respiratory (laryngeal) papillomas (17). Alternatively, anogenital warts may develop in infants within a few months of birth as a result of acquisition during vaginal delivery. Children may also develop anogenital warts due to incidental spread from cutaneous warts or from sexual abuse (18).

Cutaneous HPV lesions are common and can manifest as verruca vulgaris (HPV-2), plantar warts (HPV-1), or verruca plana (HPV-3). These common warts can manifest on any skin surface but are most commonly seen on the hands and fingers. They manifest as flesh-colored exophytic papules and nodules that are usually benign and self-limited; however, they can be annoying and difficult to eradicate.

Cutaneous warts in EV, on the other hand, can lead to major morbidity and mortality (19). EV is a rare autosomal recessive genodermatosis and was the first model of cutaneous viral oncogenesis in humans. EV patients develop disseminated warty papules and erythematous macules during childhood. These lesions progress to cutaneous carcinomas in adulthood in approximately one-half of these patients (Fig. 3). At least 19 HPV types are associated with EV. Several of these genotypes have oncogenic potential, most notably HPV-5 and HPV-8 (20). Nonsense mutations in the adjacent novel genes *EVER1* and *EVER2*, which encode integral membrane proteins in the endoplasmic reticulum (21), are associated with EV. Oncogenic HPV genotypes in EV appear to be necessary but not sufficient for malignant transformation. The most important cofactor, in the case of EV, is UV irradiation, which is illustrated by the fact that the highest incidence of carcinomas in EV patients is in areas of greatest sunlight exposure (22).

FIGURE 3 Multiple primary squamous cell carcinomas associated with human papillomavirus 8 (HPV-8) on the forehead of a man with enterovirus (EV).

Diagnosis

Often no laboratory tests are carried out if, by clinical appearance, the lesion is presumed to be HPV related and benign. When biopsies of verrucae are carried out, the following general patterns may be observed in tissues: acanthosis, papillomatosis, hyperkeratosis, parakeratosis, and prominent and often thrombosed dermal capillary vessels. Koilocytes, large keratinocytes with an eccentric, pyknotic nucleus surrounded by a perinuclear halo, are often observed. Frequently, a biopsy is conducted to determine if the lesion is dysplastic or neoplastic. In the general population, such biopsies would most likely be taken in the anogenital region. In this population, dysplastic or neoplastic lesions are most frequent on the cervix and therefore would be detectable via cytopathology taken with the Pap smear.

Immunohistochemical staining for HPV capsid antigens provides more specific detection of HPV. Because dysplastic or neoplastic lesions contain few, if any, capsid antigens, this method may give false-negative results with such lesions. HPV cannot be readily grown in tissue culture, and serology is not routinely useful; thus, the only specific method of diagnosing HPV is via DNA or RNA detection methods. The Hybrid Capture 2 High-Risk HPV DNA test and the Cervista HPV High-Risk DNA test detect presence of 13–14 oncogenic HPV types, whereas the Cervista HPV 16/18 DNA test only detects oncogenic HPV-16 and HPV-18. The Digene HC2 HPV DNA test detects 13 oncogenic or five nononcogenic HPV types. The Cobas 4500 test detects 14 oncogenic HPV DNA types and can detect individual types HPV-16 and HPV-18, whereas the APTIMA HR HPV test detects 14 oncogenic HPV types of HPV mRNA (23).

Verrucae are usually clinically evident, and they may resemble seborrheic keratoses, nevi, or acrochordons. Verrucae planae may mimic papules of lichen planus. Condyloma acuminatum must be differentiated from condyloma latum, the skin lesion associated with secondary syphilis. Bowenoid papulosis can be confused with lichen planus, psoriasis, seborrheic keratoses, or condyloma acuminata (15). Benign verrucae also must be differentiated from dysplastic and neoplastic lesions.

Treatment

Treatment for most benign verrucae consists of surgery, cryotherapy, or topical chemotherapy. In each case, the objective is to eradicate the lesion and allow the immune system to hold latent HPV in surrounding (normal-appearing) tissue in check so as to prevent recurrences. Surgical therapy includes simple excision, electrodesiccation, and removal with a CO_2 laser. Cryotherapy involves application of liquid nitrogen, which induces dermal and epidermal cellular necrosis. Topical chemotherapy options include podophyllin resin, purified podophyllotoxin, 5-fluorouracil, retinoic acid, cantharidin, salicylic acid, lactic acid, bichloroacetic acid, and trichloroacetic acid (24). Selection of the most appropriate therapy depends on the size and location of the wart, as well as on the history of previous therapies.

IFN-α is also approved for treatment of condyloma acuminatum. Combinations of IFN and other treatments do not appear to be more effective than other treatments used alone. IFN is costly and inconsistently effective and should not be considered a primary treatment (24). The Toll-like receptor 7 (TLR7) agonist imiquimod has been shown to be very effective for condyloma acuminatum (25). It is applied topically by the patient and produces minimal local inflammation and no systemic side effects. Its mode of action is via induction of endogenous IFN-α as well as a host of other cytokines. In contrast to therapies without antiviral or immunomodulatory mechanisms of action, a very low rate of recurrence is observed following clearance of condyloma acumination with imiquimod. Use of imiquimod in conjunction with surgical treatment may be even more effective. One retrospective study found that the rate of recurrent anogenital warts was much lower in patients treated with a 16-week course of imiquimod (5% cream) monotherapy and surgical excision of residual warts than in those treated with surgical excision alone (26).

Prevention

Two vaccines—Cervarix and Gardasil—protect against the two HPV types (HPV-16 and HPV-18) that cause 70% of cervical cancers, 80% of anal cancers, 60% of vaginal cancers, and 40% of vulvar cancers (27). These vaccines also protect against precancerous cervical lesions, most HPV-induced oral cancers, and other rare genital cancers. Gardasil also protects against the two HPV types (HPV-6 and HPV-11) that cause 90% of genital warts. Both vaccines are given in a series of three shots over 6 months. HPV vaccination is recommended for girls and boys at age 11 or 12 years. Women can get HPV vaccine through age 26, and men can get vaccinated through age 21. Recently, a third vaccine has been approved—Gardasil 9—which covers five additional HPV strains (HPV-31, HPV-33, HPV-45, HPV-52, and HPV-58) that are responsible for 20% of cervical cancer, in addition to the four HPV strains covered by the first-generation Gardasil vaccine. Gardasil 9 is approved for use in females ages 9–26 and males ages 9–15.

Poxviruses

Epidemiology

Smallpox was endemic throughout the world but has been eradicated by a worldwide vaccination program. Vaccination against smallpox has not been given routinely in the United States for over 30 years. Virtually all citizens are currently susceptible to variola because vaccination is believed to be protective for only 5–10 years (28). Currently, smallpox virus is known to exist in only two laboratories: the Centers for Disease Control and Prevention in Atlanta, Georgia, and a laboratory in Novosibirsk, Russia. However, there is concern that the virus exists elsewhere and could be used in biological warfare. Viral transmission is primarily via respiratory droplets. Historically, the incidence of infection was highest in the winter and early spring because aerosolized variola virus survives better at lower temperatures and low humidity levels (29).

Monkeypox has historically been significant only in sub-Saharan Africa, where it was recognized as a distinct disease in 1970, despite its presumable existence for thousands of years. Early studies suggested that most cases occurred in children under age 10 and were associated with animal contact, although secondary human-to-human transmission did occur. The first documentation of monkeypox in the Western Hemisphere was a cluster of cases in the U.S. Midwest in 2003. This outbreak was spread by prairie dogs that apparently acquired the virus from Gambian rats when housed with them at a distribution center in Illinois (30). Person-to-person spread of monkeypox through close contact with infected individuals appears to occur inefficiently. Vaccination with vaccinia virus is protective against monkeypox (31).

MC is the most prevalent poxvirus infection and commonly occurs on the trunk in children and as a sexually transmitted disease in the genital area of adults. MC spreads primarily by direct skin-to-skin contact, including autoinoculation; spread via fomites can also occur. Most children with MC are healthy and younger than 8 years of age. Fewer than 5% of children in the United States have clinical evidence of MC virus infection (32). MC virus most commonly infects individuals 15–29 years old (32, 33). Infection with MC virus occurs at increased rates among immunocompromised individuals, and the prevalence of MC among HIV-positive patients is 5–8% (33). The occurrence of MC virus infection in HIV-positive patients has decreased significantly since the introduction of highly active antiretroviral therapy (HAART). Other immunodeficient states, including systemic corticosteroid use and perhaps atopic dermatitis, can also predispose to MC virus infection.

Clinical Features

Smallpox. Following viremic dissemination, smallpox virus replicates in the epidermis and mucosae. It is spread not only via direct skin contact and fomites but also by respiratory transmission. Preceding the development of skin lesions, patients typically experience 3 days of apprehension, sudden prostrating fever, severe headache, back pain, and vomiting. Erythematous macules then develop and progress to tense, deep-seated papules and vesicles (Fig. 4). The vesicles are followed by pustules, then crusts, and finally scar formation. The rash appears in a centrifugal distribution with all lesions in the same stage of development. Overall, the mortality rate with smallpox is approximately 30%, but the hemorrhagic form results in almost 100% mortality even before development of skin lesions. Variola minor, caused by a less virulent

FIGURE 4 Variola major (smallpox).

strain of variola virus, has similar clinical manifestations but lesser severity and mortality (<1%) (34).

When smallpox was epidemic, it was occasionally confused with chickenpox, dengue fever, or enterovirus infections. An important distinguishing feature between smallpox and chickenpox is that the crops of lesions are in the same stage of development in smallpox infection, whereas lesions occur in different stages of development in chickenpox. The hemorrhagic form of smallpox has to be distinguished from other viral hemorrhagic exanthems, coagulation disorders, typhus, and meningococcal septicemia.

Vaccinia. Vaccination with vaccinia virus is no longer routine but is still used in certain target populations. The live virus vaccine occasionally leads to serious complications, including bacterial superinfections, vaccinia necrosum, generalized vaccinia, eczema vaccinatum, erythema multiforme, accidental inoculation, and encephalitis (35). These manifestations are discussed in Chapter 18.

Monkeypox. The clinical picture of human monkeypox virus infection resembles that of smallpox. There is a 10- to 14-day incubation period followed by a prodromal illness consisting of fever, malaise, and lymphadenopathy. After 1–3 days, patients break out in a maculopapular rash that usually begins on the trunk and spreads peripherally. Lesions can occur on the palms and soles and on mucous membranes. The mortality rate is 10%, and death usually occurs during the second week of the illness (31).

Monkeypox and smallpox have very similar clinical manifestations. One distinguishing feature clinically is lymphadenopathy, which is common in the prodromal phase of monkeypox virus infection but not smallpox (31). However, these poxviruses cannot be readily identified from one another except by PCR assay (34).

Molloscum Contagiosum. The incubation period of MC is 2–6 weeks. It manifests as 3- to 6-mm skin-colored dome-shaped papules with a central umbilication. Although four different strains of MC virus (I through IV) have been identified (based on restriction endonuclease digestion patterns), all strains produce similar clinical pictures. MCV I is responsible for the vast majority of infections in immunocompetent hosts in the United States. Clinical presentations of MC often follow one of two patterns in immunocompetent individuals: widespread papules on the trunk and face of children, transmitted by direct skin-to-skin (nonsexual) contact, or genital papules in adults, spread by sexual contact. In either case it is unusual to see more than 15 lesions in an individual patient. In immunocompromised persons, especially those who are HIV positive, MC can manifest with thousands of papules and be a major source of morbidity (**Fig. 5**); HIV-positive patients also commonly have facial involvement as well as increased likelihood of bacterial superinfection and treatment resistance (32).

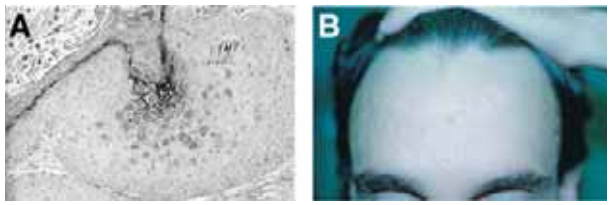

FIGURE 5 (a) Photomicrograph of molluscum contagiosum (MC) demonstrating central umbilication and epidermal hyperplasia containing intracytoplasmic inclusions (Henderson-Paterson bodies) compressing nuclei. (b) Disseminated molluscum contagiosum in an AIDS patient.

A number of entities can mimic MC in the healthy host, such as warts, basal cell carcinomas, and lichen planus. In HIV-positive patients, MC must be distinguished from cutaneous cryptococcal infection (36).

Orf. Orf, contagious ecthyma, is a less common poxvirus infection that is transmitted from sheep, goats, and other animals to the hands of humans (**Fig. 6**). Orf manifests as cutaneous nodules averaging 1.6 cm in diameter associated with regional lymphadenopathy, lymphangitis, and fever. Lesions spontaneously progress through six stages, resulting in healing in about 35 days (37).

Milker's Nodules. Milker's nodules are caused by parapoxvirus. Clinically similar to orf, the lesions result from manual contact with teats of infected cows and have an incubation period of 4–7 days. Also similar to orf, the nodules heal in 4–6 weeks after progressing through six clinical stages (38).

Orf and milker's nodules can mimic one another or can be confused with pyogenic granulomas, sporotrichosis, or atypical mycobacterial infection.

Treatment

For patients with smallpox or disseminated vaccinia, management of symptoms and prevention of bacterial superinfection are paramount. See Chapter 18 regarding antiviral therapy. There is no proven effective treatment for monkeypox, but vaccination is highly protective.

MC is a self-limited condition, but resolution may take 6 months to 5 years. Therefore, most physicians recommend treatment, particularly for genital lesions, to reduce the risk

FIGURE 6 Nodular stage of orf on the hand of a shepherd from Mexico.

of spread and patient discomfort. Treatment options include local excision by electrocautery, curettage, or cryotherapy and chemical ablation via application of trichloroacetic acid or podophyllin. Patients may opt to treat their lesions at home by self-administering topical treatments such as podophyllotoxin, retinoic acid, or imiquimod cream (33). Recurrences are common in immunocompromised persons. Lesions of orf or milker's nodules can be removed via excision and cautery, but this is usually not necessary as spontaneous resolution can be expected in approximately 6 weeks.

Human Herpesviruses—Herpes Simplex Virus (HSV-1, HSV-2)

Epidemiology and Clinical Manifestations

HSV-1 is the primary cause of oral herpes or herpes labialis and an increasing number of genital infections. The virus is typically transmitted via direct inoculation of the skin and mucous membranes. Herpes labialis is extremely common, with up to 90% of adults having serologic evidence of HSV-1 infection (39). While the majority of primary infections with HSV-1 are asymptomatic, the virus remains dormant in the host's neuronal ganglia and can reactivate to produce recurrent symptomatic disease. Recurrent episodes occur in up to one-third of infected individuals and can be induced by stress, trauma, menstruation, fever, colds, and ultraviolet (UV) light. Upon reactivation, the virus travels down the sensory nerve, producing prodromal sensations of pruritus or tingling. When the virus reaches the skin, grouped vesicles with surrounding erythema form on or near the vermillion border of the lip. There is often regional lymphadenopathy and occasionally fever, headache, and malaise. Vesicles ulcerate, crust, and resolve in 2–4 weeks, frequently with postinflammatory hypo- or hyperpigmentation.

At least 50 million individuals in the United States, or one in four adults, are estimated to be seropositive for HSV-2 (40). HSV-2 is the primary cause of genital herpes or herpes genitalis. As with HSV-1, most primary infections with HSV-2 are asymptomatic, with only 10–25% of seropositive individuals indicating a history of genital ulcers (40). Primary HSV-2 infection classically manifests with widespread genital vesicles and ulcers with surrounding erythema (Fig. 7). There may be associated edema, pain, inguinal lymphadenopathy, discharge, dysuria, malaise, fever, and photophobia. These signs and symptoms typically occur within 3–14 days of sexual contact with an infected individual. Viral shedding from active lesions lasts up to 14 days in women and approximately 10 days in men (41); however, HSV-2-seropositive individuals can still shed the virus and infect others when no lesions are present. Symptoms are typically more severe in women than in men, and skin lesions often require 3–4 weeks for complete healing.

Similar to HSV-1, recurrences of HSV-2 may be triggered by a variety of factors, such as emotional or physical stress or mild trauma. Recurrent episodes of HSV are usually less severe than initial outbreaks and often heal in 7–10 days without therapy. Men suffer 20% more recurrences than women, which may contribute to the higher rate of herpes transmission from men to women. In immunocompromised individuals, HSV recurrences may be chronic and result in significant morbidity if untreated.

Up to 30% of first-episode genital herpes is due to HSV-1, which is often attributable to orogenital contact. Genital herpes due to HSV-1 is usually less severe than disease due to HSV-2. In addition, genital herpes due to HSV-1 recurs less frequently than HSV-2-associated disease.

FIGURE 7 First-episode genital herpes due to herpes simplex virus 2 (HSV-2) in a man who gave a history of always using condoms during sex.

In addition to herpes labialis and genitalis (Fig. 8), HSV-1 and HSV-2 can also cause gingivostomatitis, herpetic whitlow (Fig. 9), HSV gladiatorum, neonatal herpes, herpetic keratoconjunctivitis, aseptic meningitis, and herpes encephalitis. Complications of HSV infection include erythema multiforme (Fig. 10) and eczema herpeticum due to autoinoculation of the virus onto areas of atopic dermatitis. In immunocompromised patients, infection with HSV-1 or HSV-2 can lead to widespread local infection, as well as disseminated cutaneous and visceral infection.

Neonatal herpes is caused by HSV-1 or HSV-2 transmitted *in utero*, intrapartum, or postnatally. The details of newborn HSV infection are described in Chapter 19.

Diagnosis

Diagnosis of HSV-1 and HSV-2 infection is often made clinically but can be confirmed by viral culture, Tzanck

FIGURE 8 Recurrent herpes simplex virus 2 (HSV-2) infection of the buttock.

FIGURE 9 Herpes whitlow due to herpes simplex virus 2 (HSV-2) in a health care worker following a puncture wound from a needle used to culture genital herpes.

smear (Fig. 11), PCR, serology, and antigen detection. HSV-1 and HSV-2 typically grow readily within 1 to 2 days in cell culture (42), but real-time HSV PCR assays have emerged as a more sensitive method to confirm HSV infection in clinical specimens obtained from genital ulcers and mucocutaneous sites. Because the prognosis is different for herpes genitalis caused by HSV-1 versus HSV-2, differentiating between the two is important for patient counseling. Serology not only can differentiate between HSV-1 and HSV-2 but also can be helpful in distinguishing primary genital herpes with a predominance of immunoglobulin M (IgM) antibodies from nonprimary genital herpes with a high proportion of IgG.

The differential diagnosis for vesicular lesions associated with HSV infection includes contact dermatitis, bullous impetigo, and insect bites. For lesions that are located specifically in the orolabial region, aphthous stomatitis, HFMD, and herpangina should be considered. In addition, erythema multiforme should be considered, as HSV is the most common identifiable etiologic agent (43). The differential for lesions in the genital region includes urethritis, urinary tract infections, tinea cruris, and vaginitis.

Treatment and Prevention

HSV-1 and HSV-2 are commonly treated with acyclic nucleoside analogues that block viral DNA polymerase, such as acyclovir. Topical, oral, and intravenous acyclovir are available, although the topical formulation is rarely used due to its limited penetration of the stratum corneum. Oral acyclovir, especially if started early, accelerates the rate of

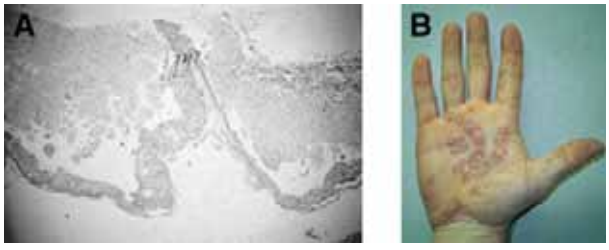

FIGURE 10 (a) Photomicrograph of erythema multiforme associated with herpes simplex virus 1 (HSV-1), showing subepidermal vesicles, necrotic keratinocytes, and balloon cell degeneration. (b) Erythema multiforme following an outbreak of herpes labialis.

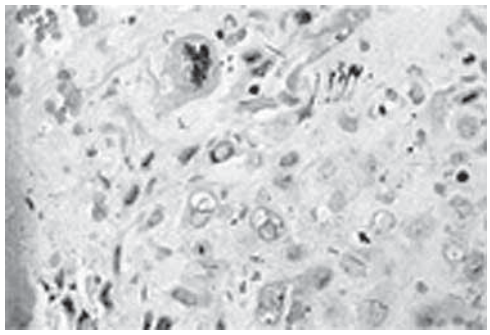

FIGURE 11 Photomicrograph of an ulcer resulting from herpes simplex virus 2 (HSV-2), in which multinucleated giant cells with eosinophilic intranuclear inclusions can be seen.

lesion crusting in oral and genital herpes (44). In patients with severe or frequent recurrences or with ocular HSV, suppressive therapy with acyclovir has been shown to decrease the recurrence rate by 50% (44). Acyclovir has also been shown to reduce asymptomatic viral shedding of HSV-2 by 95% (45).

In immunocompromised patients with mucocutaneous HSV, especially in disseminated disease, intravenous acyclovir is favored. Intravenous acyclovir is also used for infection in neonates, eczema herpeticum, and herpes encephalitis (45). Foscarnet is approved for the treatment of acyclovir-resistant HSV infections.

Currently, valacyclovir and famciclovir along with acyclovir are the mainstay drugs for treating as well as suppressing genital herpes. One study demonstrated a 50% decrease in HSV-2 transmission with once-daily valacyclovir (40). Famciclovir has also been shown to be an effective, well-tolerated option for the suppression of genital herpes among individuals with multiple recurrences (46).

The development of an effective vaccine against HSV-1 and HSV-2 has proven challenging, and no vaccine is currently available. Two studies of a prophylactic glycoprotein D2 alum/monophosphoryl lipid A (MPL) vaccine demonstrated prevention of genital herpes disease in 73% (first study) and 74% (second study) of seronegative women whose regular sexual partner had a history of genital herpes (47). A second prophylactic vaccine (ICP10DPK, AuRx) was shown to prevent recurrent disease in 44% of immunized subjects and to reduce the frequency and severity of recurrences in subjects that were not fully protected (48). A more recent study of HSV-2 glycoprotein D has shown 58% efficacy against HSV-1, but was not efficacious against HSV-2 (49).

Varicella-Zoster Virus

Epidemiology and Clinical Manifestations

VZV, or human herpesvirus type 3 (HHV-3), is a highly prevalent pathogen, with 98% of the adult population in the United States having serologic evidence of previous infection. VZV causes two distinct diseases: primary varicella (chickenpox) and herpes zoster (shingles). Before the introduction of a vaccine in 1995, 3–4 million cases of varicella leading to approximately 11,000 hospitalizations and 100 deaths were reported each year (50). Transmission occurs via direct contact or airborne droplets. Currently, more than 1,000,000 cases of herpes zoster occur each year in the United States; however, the use of the VZV vaccine will significantly alter the epidemiology of both syndromes.

Primary varicella typically manifests in younger children as low-grade fever, malaise, and disseminated pruritic vesicles classically described as "dewdrops on a rose petal." Skin lesions first appear on the face and trunk as erythematous macules and rapidly progress over 12–14 hours to papules, vesicles, pustules, and crusts (Fig. 12). Most of the lesions are seen centrally and on the proximal extremities. Vesicles also appear on mucous membranes, but they erode rapidly to form shallow, painful ulcers. Due to the rapid evolution of successive crops of lesions, varicella is characterized by the simultaneous presence of lesions in all stages of development within the same anatomic region. In older children and adults, the exanthem is often preceded by up to 3 days of prodromal symptoms, including headache, myalgias, anorexia, nausea, and vomiting.

The most common cutaneous complication of varicella in young, immunocompetent individuals is scarring, which is often secondary to bacterial superinfection with *Staphylococcus aureus* or *Streptococcus pyogenes*. In adults and immunocompromised individuals, significant morbidity and occasional mortality can result from complications of VZV infection, including myelitis, large vessel granulomatous arteritis, encephalitis, varicella pneumonia, and varicella hepatitis (51). Maternal infection with VZV in the first trimester is associated with a 2% risk of congenital malformations, such as intrauterine growth retardation, limb hypoplasia, cataracts, chorioretinitis, microcephaly, cortical atrophy, and skin lesions (52). The skin lesions typically consist of areas of hypertrophic scarring with induration and erythema located especially on the extremities.

In 20% of immunocompetent individuals and in up to 50% of immunocompromised individuals, VZV reactivates years or even decades later to produce herpes zoster. Although in most cases the exact trigger for reactivation is unknown, advancing age is an important factor, with the majority of cases occurring in individuals over 50. A family history of shingles also appears to increase the risk of herpes zoster (53). Upon reactivation, the virus spreads down the sensory nerves, and transient virema occurs with associated prodromal symptoms of pain, fever, regional lymphadenopathy, and malaise (54). After a few days to weeks of pain, vesicles appear along the distribution of the sensory nerve (Fig. 13). Although vesicles generally occur only along one dermatome, it is not unusual for a few lesions to appear in neighboring dermatomes. The predilection for zoster to appear in certain anatomic regions (face and trunk) usually corresponds to the areas most affected by primary varicella. After a few days, the vesicles become pustules, and within 1–2 weeks the pustules become crusts. The skin lesions found in herpes zoster can shed VZV and cause primary varicella in seronegative individuals.

Although scarring can occur, particularly in darker-skinned individuals, cutaneous complications of herpes zoster are rare. The most prevalent complication is postherpetic neuralgia, which is defined as persistent pain for more than 8–12 weeks after initial rash appearance (51). The pain, which may be extremely severe, can last for months to years and be highly resistant to treatment. Other complications of herpes zoster include vision impairment or blindness with involvement of the ophthalmic branch of the trigeminal nerve and painful facial paralysis (Ramsay Hunt syndrome) with involvement of the facial and auditory nerves (55). Rarely, sensory defects, motor paralysis, and encephalomyelitis can occur (56, 57). Dissemination of herpes zoster, defined as more than 20 vesicles outside the primary and adjacent dermatomes, is rare in healthy hosts but can occur in up to 40% of severely immunocompromised individuals. Cutaneous dissemination may be a marker of visceral involvement (liver, lungs, and the CNS) and therefore can herald significant morbidity and mortality.

Diagnosis

Varicella and herpes zoster are often diagnosed clinically on the basis of the characteristic vesicular lesions, which are widespread in chickenpox (varicella) or restricted in a dermatomal pattern with associated neuritis in shingles (herpes zoster). However, laboratory confirmation is useful in circumstances where the clinical presentation is atypical and challenging, especially in immuncompromised patients. The diagnostic techniques include viral culture, direct immunofluorescence testing, serology testing, and PCR assay, which is the most sensitive test. Virus isolation by culture is insensitive, requires prolonged incubation, and associated with low yield (approximately 60–75%) when compared with PCR testing (58).

FIGURE 12 Primary varicella in an adult Japanese man.

FIGURE 13 Thoracic herpes zoster in an otherwise healthy man.

Treatment

For most children, primary varicella is a benign, self-limited disease and treatment is largely supportive. However, pregnant women and neonates are at considerable risk for morbidity and mortality and are treated with antivirals such as intravenous acyclovir (58, 59). Treatment for herpes zoster includes antivirals and analgesics for pain control. Acyclovir, valacyclovir, and famciclovir are FDA approved for the management of acute herpes zoster, although they may not decrease the rate of development of postherpetic neuralgia (58). Antiviral therapy is imperative in patients with herpes zoster that involves the ophthalmic branch of the trigeminal nerve to prevent vision loss (58). Pain management is especially difficult with conventional analgesics in herpes zoster patients who develop postherpetic neuralgia. Tricyclic antidepressants, selective serotonin and norepinephrine reuptake inhibitors (duloxetine and venlafaxine), opioid, calcium channel α_2-δ ligands (gabapentin and pregabalin), topical capsaicin, and topical lidocaine have been shown to reduce the pain associated with postherpatic neuralgia (60). Of these medications, only gabapentin, pregabalin, 5% lidocaine patch, and 8% capsaicin patch have been approved by the FDA specifically for the treatment of postherpetic neuralgia (61). Adding gabapentin to an antiviral in patients with acute herpes zoster appears to reduce significantly the incidence of postherpetic neuralgia (62). In January of 2011, the FDA approved Gralise™ as a once-daily medication for the treatment of postherpetic neuralgia (63). Gralise is an extended-release form of gabapentin that not only has been shown to decrease postherpetic neuralgia pain scores significantly, but may also be associated with fewer side effects than its immediate-release counterpart. In 2012 the FDA approved Horizant™, gabapentin enacarbil, for the once-daily therapy of postherpetic neuralgia.

Prevention

Vaccines are currently available for prophylaxis of varicella (Varivax vaccine) and herpes zoster (Zostavax vaccine). The live attenuated viral vaccine (Oka strain) was approved in 1995 and produces a 95% seroconversion rate (64). Significant adverse events with the vaccine are rare, with fewer than 5% experiencing a mild varicella-like disease. There has been a significant decrease in varicella since the vaccine was instituted, and vaccination will likely alter the epidemiology of herpes zoster as well (65). Children should get the first dose of the vaccine at 12–15 months and the second dose at 4–6 years. Varicella vaccine doses given to persons 13 years or older should be separated by 4–8 weeks. The VZV vaccine is contraindicated in immunocompromised individuals, in persons with a history of anaphylactic or anaphylactoid reactions to gelatin or neomycin, and in pregnant women (66). Varicella-zoster immunoglobulin is recommended for post-exposure prophylaxis in immunocompromised persons, pregnant women, and neonates born to mothers who acquired varicella a week before or up to 2 days after delivery.

In a clinical trial involving more than 38,000 adults 60 years of age or older, the vaccine was shown to reduce the incidence of herpes zoster by 51% and the incidence of postherpetic neuralgia by 67% (67). In 2006, the Advisory Committee on Immunization Practices recommended a single dose of zoster vaccine for adults 60 years of age or older, whether or not they have had a previous episode of herpes zoster (68). A subsequent clinical trial that studied Zostavax in patients aged 50 to 59 years showed that the vaccine efficacy for preventing herpes zoster was 69.8% in this age group. In 2011 the FDA approved Zostavax for patients 50 to 59 years of age (69). An adjuvanted herpes zoster subunit vaccine (HZ/su), that is currently being studied but is not yet approved, was shown to significantly reduce the risk of herpes zoster among adults who were 50 years of age or older. However, unlike Zostavax, the HZ/su vaccine efficacy was well preserved among participants who were 70 years of age or older (70).

Epstein Barr Virus

Clinical Manifestations

The EBV, or HHV-4, causes infectious mononucleosis, also known as "the kissing disease," because the virus is typically transmitted through oral secretions. Infectious mononucleosis first manifests as prodromal symptoms of malaise, headache, and fatigue, followed by fever, sore throat, and cervical adenopathy. Hepatomegaly and splenomegaly can also occur. Cutaneous manifestations of infectious mononucleosis include macules, papules, and, less commonly, erythema, vesicles, and purpura. These lesions are the result of viral replication and manifest during the first week of illness. In approximately one-third of patients, small petechiae are observed at the border of the hard and soft palates. If infectious mononucleosis is treated inappropriately with ampicillin or other penicillins, a high percentage of patients develop erythematous macules and papules over the trunk and extremities (71**).** These lesions persist for about 1 week, followed by desquamation.

EBV has a pathogenic role in the development of many cancers, especially in immunocompromised individuals; these cancers include Burkitt's lymphoma, Hodgkin's disease, nasopharyngeal carcinoma, and posttransplantation B-cell lymphoma (51). EBV expression has also been detected in cutaneous T-cell lymphomas, including mycosis fungoides, although its role is yet to be determined (72). Mycosis fungoides initially manifests as annular pink scaly patches that over time develop into patches and plaques that may resemble psoriasis. Finally, large irregular tumors form that may ulcerate. EBV DNA has also been detected in epithelial cells of oral hairy leukoplakia, an oral lesion closely associated with HIV infection (73) (Fig. 14). Finally, Gianotti-Crosti syndrome, manifested as symmetric, nonpruritic, lichenoid papules of the face, limbs, and buttocks, has also been associated with primary EBV infection (74) (Fig. 15).

Diagnosis

Diagnosis is usually made through the detection of specific antibodies to EBV (see Chapter 24: Epstein-Barr Virus.

FIGURE 14 Oral hairy leukoplakia associated with Epstein-Barr virus (EBV) in an AIDS patient.

FIGURE 15 Papules of Gianotti-Crosti syndrome associated with Epstein-Barr virus (EBV).

Authors: Katherine Luzuriaga, John L. Sullivan). In particular, the monospot test, which detects heterophile antibodies, is used to diagnose infectious mononucleosis. This test is not commonly used for children due to its high false-negativity rate. Peripheral smears can support the diagnosis if greater than 10% atypical lymphocytes are noted.

The differential diagnosis for the classic symptoms of sore throat, malaise, and lymphadenopathy associated with infectious mononucleosis includes streptococcal pharyngitis and other viral causes of pharyngitis. Acute HIV syndrome can also manifest as malaise, lymphadenopathy, and nonspecific mucocutaneous manifestations. Acute CMV infection can cause infectious mononucleosis similar to EBV mononucleosis. The cutaneous manifestations of infectious mononucleosis may resemble a number of nonspecific viral exanthems. However, if the findings are preceded by the recent administration of ampicillin, the probability of EBV infection is high.

Treatment

Treatment for infectious mononucleosis due to EBV is largely supportive. Antivirals, such as acyclovir, and corticosteroids have not been shown to be effective (75, 76). The development of anti-B-cell antibodies, such as rituximab, has greatly enhanced the therapeutic options for EBV-associated cancers (77, 78).

Cytomegalovirus

Epidemiology and Clinical Manifestations

CMV is an ubiquitous virus that is transmitted through infectious secretions. In developing countries, close to 100% of the adult population are seropositive, while in developed countries only about 50% of adults have evidence of infection. Primary infection is usually subclinical in immunocompetent individuals, although CMV mononucleosis syndrome occurs. Symptoms include fever, fatigue, and, less commonly, lymphadenopathy, sore throat, and organomegaly. Up to one-third of patients with CMV mononucleosis develop a maculopapular generalized rash.

Primary CMV infection in pregnant women poses a serious threat to the fetus. Maternal CMV infection is considered a leading viral cause of congenital malformations, CNS injury, and hearing loss in the neonate. If primary maternal infection occurs during pregnancy, especially during the first trimester, the rate of transmission is about 40% (79). Many of these infants have clinical manifestations at birth, including intrauterine growth retardation, microcephaly, cerebral atrophy, periventricular calcifications, chorioretinitis, sensorineural hearing loss, thrombocytopenia, and hepatosplenomegaly. Cutaneous manifestations include jaundice and purpuric macules and papules, secondary to persistent dermal hematopoiesis, resulting in the clinical picture of the "blueberry muffin baby." With recurrent maternal infection during pregnancy, the risk of transmission is only about 1%, and most of these infants have clinically silent disease at birth (79).

In immunocompromised patients, in whom CMV is associated with a variety of clinical entities, including retinitis, hepatitis, and colitis, infection may be associated with a variety of skin lesions, from vesicles to verrucous plaques. The most prevalent cutaneous manifestation is ulceration, especially in the perianal area (80). These cutaneous ulcerations are the result of CMV infection of the vascular endothelium and subsequent destruction of blood vessels.

Diagnosis

CMV DNA levels in acute CMV infection can provide prognostic information for immunocompromised patients. Histology can also be beneficial in diagnosis. CMV-infected cells have characteristic intranuclear inclusions surrounded by clear halos resembling "owl's eyes."

In the neonate with congenital CMV infection, the differential diagnosis includes other congenital infections, including toxoplasmosis, rubella, HSV, syphilis, and lymphocytic choriomeningitis virus. For older patients, the differential diagnosis for CMV infectious mononucleosis includes EBV mononucleosis. The monospot test is generally negative in CMV mononucleosis.

Treatment

Ganciclovir, a nucleoside analog of guanosine, has been shown to be effective in the treatment and prophylaxis of CMV infections (81, 82). Valganciclovir, a prodrug of ganciclovir, is available orally and has significantly increased bioavailability compared to ganciclovir, with similar safety and efficacy profiles (82). Valganciclovir has been approved for the treatment of CMV retinitis in adult patients with acquired immunodeficiency syndrome (AIDS), as well as for prophylaxis of CMV disease in organ transplant recipients (83). For ganciclovir- and valganciclovir-resistant CMV infections, intravenous foscarnet or cidofovir are the drugs of choice. For further discussion, see also Chapters 12 and 22.

Human Herpesviruses—HHV-6 and HHV-7

Epidemiology and Clinical Manifestations

HHV-6 and HHV-7 are highly prevalent infections; 90% of children have serologic evidence of HHV-6 infection by 2 years of age (84). HHV-7 infection usually occurs later, with most children seropositive by 5–6 years of age (85). Transmission is through oropharyngeal secretions. Primary infection with HHV-6 is a common cause of fever, irritability, and rhinorrhea in children. One study found that primary HHV-6 infection accounted for 20% of fevers in children between 6 and 12 months of age (86). HHV-6, as well as HHV-7, is also associated with the common childhood exanthem roseola infantum (exanthem subitum or sixth disease). Roseola infantum manifests as a high fever lasting 3–5 days followed by the development of a nonpruritic, blanchable, pink, maculopapular rash on the neck and trunk. Other cutaneous manifestations include palpebral edema and lesions on the soft palate. Roseola infantum is usually self-limited but can, rarely, cause seizures and encephalitis (87).

HHV-7 is also suspected to play a role in the pathogenesis of pityriasis rosea, another acute, self-limited exanthem.

Pityriasis rosea begins with the development of a herald patch, a single plaque that is salmon-colored to red with fine scale at the periphery. The herald patch is followed by the development of pink papules and plaques in a "Christmas tree" distribution on the trunk.

Diagnosis

Diagnosis is usually made clinically but can be confirmed via serology, peripheral blood mononuclear cell culture, or PCR (86). The clinical presentation of roseola infantum and its rapid resolution distinguish it from other entities on the differential diagnosis, including drug eruptions, scarlet fever, rubella, measles, erythema infectiosum, and other viral exanthems. The differential diagnosis for pityriasis rosea includes drug eruptions, secondary syphilis, guttate psoriasis, erythema multiforme, and tinea corporis. The presence of a herald patch and the resolution of pityriasis rosea without treatment can aid in diagnosis.

Treatment

Treatment is largely supportive. There have been no controlled trials of antiviral therapy or specific recommendations for the management of HHV-6 and HHV-7 (88).

Human Herpesvirus 8

Epidemiology

The prevalence of HHV-8 infection varies significantly in populations worldwide. In the United States, less than 5% of adults have serologic evidence of HHV-8, whereas in highly endemic areas, such as Africa, more than 50% are seropositive (89). HHV-8 is predominantly shed in the saliva and to a lesser degree in semen and other body fluids. In low-prevalence areas, transmission is mainly through sexual contact, whereas in high endemic areas, transmission is typically from mother to child and between siblings (89). HHV-8 is associated with the development of Kaposi's sarcoma (KS) in both HIV-infected and HIV-negative persons (90, 91).

KS is the most common AIDS-associated malignancy in the developed world and one of the most common cancers in developing nations. KS in HIV-negative patients is rare. Two groups are at risk to develop non-AIDS related KS: elderly men mainly of Mediterranean origin and persons with iatrogenic immunosuppression.

Clinical Manifestations

KS are vascular neoplasias that initially present as deep red-purple macules (Fig. 16). The macules evolve into papules, plaques, and tumors that can be pink, red, purple, or brown. Classically, the lesions begin on the feet and hands and spread proximally. There is often associated lymphedema, especially of the lower extremities. KS can present with oral lesions as well and involve almost any internal organ.

HHV-8 is also associated with primary effusion lymphoma and multicentric Castleman's disease. Castleman's disease is caused by the hyperproliferation of B cells forming tumors in lymph nodes throughout the body. In addition, HHV-8 DNA has also been detected in squamous cell carcinomas and other epithelial lesions in organ transplant recipients (91). The role of HHV-8 in the pathogenesis of these epithelial tumors is still unclear.

Diagnosis and Differential Diagnosis

The differential for KS includes dermatofibroma, pyogenic granuloma, hemangioma, bacillary (epithelioid) angiomatosis, melanocytic nevus, ecchymosis, granuloma annulare, stasis dermatitis, and insect bites (92). Both PCR and serologic markers can aid in diagnosis, although skin biopsy is usually necessary for confirmation (93, 94) (Fig. 17).

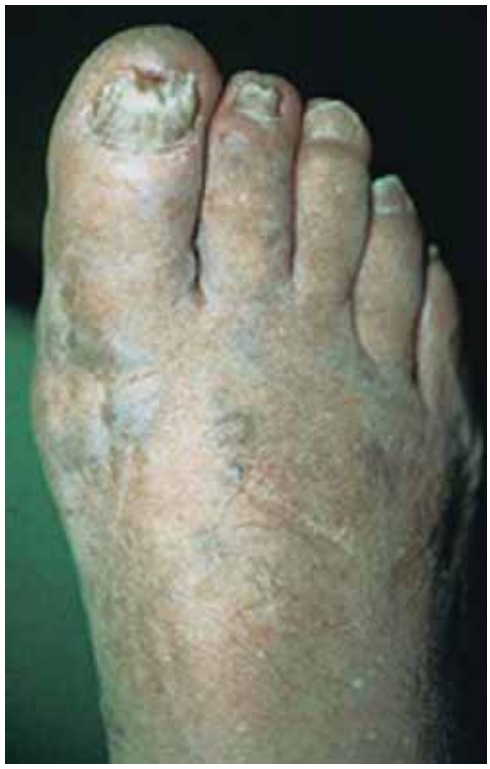

FIGURE 16 Kaposi's sarcoma associated with human herpesvirus 8 (HHV-8) in an HIV-negative elderly Italian man.

Treatment

Although ganciclovir, foscarnet, and cidofovir have ***in vitro*** activity against HHV-8 and limited studies indicate these agents may be associated with reduced KS disease progression or lesion regression, larger and more definitive studies are needed to determine whether antiviral therapy has a useful role in managing HHV-8-associated diseases. KS regression has been documented after ganciclovir or foscarnet therapy, although one study indicated cidofovir was ineffective (95).

The use of intravenous (i.v.) ganciclovir or oral valganciclovir is an option for treatment of multicentric Castleman's disease. A 3-week course of twice-daily i.v. ganciclovir or oral valganciclovir was associated with remissions in multicentric Castleman's disease in one report, and a combination of valganciclovir and high-dose zidovudine given for 7–21 days led to durable clinical remissions of the disease (96, 97). Rituximab also is an effective alternative to antiviral therapy in the treatment of multicentric Castleman's disease, although up to one-third of patients treated with rituximab may have subsequent exacerbations or emergence of KS. (27, 28,98–101)

B Virus (Herpesvirus Simiae)

An animal herpesvirus, B virus (herpesvirus simiae), can also rarely cause human disease, most significantly a fatal encephalomyelitis. This virus usually infects humans following a bite or scratch from a macaque monkey. Erythema, induration, and vesicles develop at the inoculation site

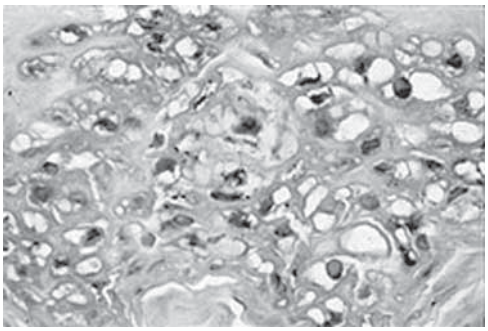

FIGURE 17 Photomicrograph of Kaposi's sarcoma associated with human herpesvirus 8 (HHV-8), demonstrating neovascularization, endothelial proliferation, and plump hyperchromatic atypical nuclei lining vascular spaces.

and are followed by fever, lymphangitis, lymphadenopathy, gastrointestinal symptoms, and myalgias. These symptoms are followed by rapid progression to the neurologic signs and symptoms of encephalomyelitis (102). Although many nonhuman herpesviruses exist, B virus is of particular importance due to the high mortality rate in infected humans (103). Diagnosis is typically made through viral culture. The virus can be recovered from vesicular skin lesions at the point of inoculation, as well as from vesicles following reactivation of the latent B virus.

Parvovirus B19

Epidemiology

Parvovirus B19 infection is common worldwide and occurs both sporadically and as epidemics. Parvovirus B19 has been recognized since the 1980s as the cause of erythema infectiosum (fifth disease) (104). This syndrome presents most commonly in children 4–10 years of age and often in epidemics in late winter and early spring (105). Viremia appears 6–14 days after a susceptible patient contracts parvovirus B19 via the respiratory route, but the rash appears 17–18 days following infection. Approximately 60–70% of adults are parvovirus B19 IgG seropositive, with prevalence rates increasing with age (106). The rate of primary parvovirus B19 infection in adults is much higher in those who are immunocompromised. Parvovirus B19 typically affects 1–5% of pregnant women, but higher attack rates (up to 20%) occur during an epidemic. Infection of a pregnant woman, particularly during the first or second trimester, can lead to nonimmune hydrops fetalis and fetal death (107).

The virus is spread by respiratory droplets, but nosocomial infections have been described (108). Parvovirus B19 has also been transmitted by blood products, especially pooled factor VIII and factor IX concentrates. Since January of 2002, producers of plasma derivatives have voluntarily instituted quantitative measurements of B19 DNA to reduce the risk of iatrogenic transmission. Iatrogenic transmission of parvovirus B19 via blood products continues to occur in part because the virus's small size, heat resistance, and high viral load make it difficult to eradicate from blood and plasma derivatives (109).

Clinical Features

Erythema infectiosum begins with nonspecific symptoms approximately 4–14 days after exposure to parvovirus B19 but can begin as late as 21 days after exposure (110). Confluent erythematous, edematous plaques appear on the cheeks with circumoral pallor after about 2 days of low-grade fever, headache, and coryza. The rash gives the cheeks a "slapped" appearance (Fig. 18) and is accompanied by continuation of the above-mentioned symptoms and the appearance of cough, conjunctivitis, pharyngitis, malaise, myalgias, nausea, diarrhea, and occasional arthralgias. After 1–4 days, the facial rash fades coinciding with the appearance of erythematous macules and papules with a reticulated pattern on the extensor surfaces of the extremities, neck, and trunk. The rash can be pruritic and usually lasts for 1–2 weeks, but it can persist for months. The rash may be evanescent, and recurrences can be provoked by exposure to sunlight, heat, emotional stress, or exercise. Patients with erythema infectiosum appear to be infectious only before the appearance of the rash, as parvovirus B19 is usually not found in respiratory secretions or in the serum after the appearance of cutaneous manifestations. Parvovirus myocarditis (111) and heart failure (112) have followed fifth disease in a small number of cases. Parvovirus infection has been associated with severe but self-limited hepatitis in a few children (113).

The gloves-and-socks syndrome, an exanthem localized to the hands and feet, with edema, erythema, paresthesia, and pruritus, has also been linked to parvovirus B19 (114). Chronic fatigue syndrome may follow infection with parvovirus B19 (115). Meningitis, encephalitis, and a variety of neurologic complications may occur with fifth disease and parvovirus infection (116). In adults, primary parvovirus B19 infection is often associated with an acute arthropathy without rash. Other clinical presentations of parvovirus B19 infection, uncommonly accompanied by rash, include transient aplastic crisis in patients with chronic hemolytic anemia, parvovirus-related chronic anemia in immunocompromised patients, and nonimmune fetal hydrops (117).

Diagnosis

Detection of serum IgM directed to parvovirus B19 indicates recent infection. The serum IgM levels start to decline after 1 month, but IgM is still detectable for 6 months after infection. Parvovirus B19-specific IgG can be detected 1 week following infection and persists for years. Severely immunocompromised hosts may fail to mount antibody responses. PCR is available for detection of the virus.

The macular and papular stages of erythema infectiosum must be differentiated from drug eruptions, bacterial infections (such as scarlet fever and erysipelas), and other viral infections (such as those due to enteroviruses and rubella, measles, and roseola viruses).

FIGURE 18 Erythema infectiosum (fifth disease) associated with parvovirus B19.

Treatment

Because erythema infectiosum is a self-limited and mild illness, no treatment is usually indicated. No specific antiviral therapy exists for parvovirus B19, but intravenous immunoglobulin with specific neutralizing antibody is useful in treating infections in immunocompromised hosts (118, 119). Nonsteroidal anti-inflammatory drugs are often used to relieve arthralgias and arthritis.

RNA VIRUSES

Enteroviruses

Epidemiology

Enteroviruses are highly contagious and are typically transmitted by human oral–oral and fecal–oral routes. Direct contact with fluid from cutaneous and ocular lesions, fomites, and contaminated water sources may also be mechanisms of transmission. The gastrointestinal tract may remain infected, thereby releasing virus into the feces for days, weeks, or even months after initial infection, thus allowing the potential for spread (120, 121).

Enterovirus infections occur worldwide. It has been estimated that each year there are approximately 10–15 million symptomatic enterovirus infections in the United States (122). These infections tend to have a seasonal preference for summer and fall, and a higher incidence in children younger than 10 years of age has been reported (123).

Pathogenesis

After infection via the buccal mucosa, pharynx, or gastrointestinal tract, the virus travels to regional lymph nodes, and a secondary viremia results in the virus seeding secondary locations, including mucocutaneous sites among others, resulting in intraepidermal vesicles containing neutrophils, mononuclear cells, and proteinaceous eosinophilic material. The subvesicular dermis is edematous and contains a perivascular polymorphous infiltrate composed of lymphocytes and neutrophils.

Clinical Features

Enteroviruses can cause a variety of clinical manifestations. Common mucocutaneous presentations (Table 1) and less common manifestations (Table 2) are recognized. While a variety of enteroviruses, particularly coxsackieviruses, cause mucocutaneous manifestations, the two most distinctive syndromes are HFMD and herpangina.

Hand-Foot-and-Mouth Disease. HFMD is a mucocutaneous manifestation that usually affects persons in their preteen and teenage years (124). Serotypes CVA16 and EV71 are responsible for most epidemic cases of HFMD, but occasionally HFMD may be associated with CVA4–CVA7, CVA9, CVA10, CVB1–CVB3, CVB5, and echovirus 4 (125). EV71 is a major public health issue across the Asia-Pacific region and beyond. Since the late 1990s, large EV71 epidemics with significant numbers of fatalities have been reported in Malaysia, Taiwan, Mainland China, and other Southeast and East Asian countries (126).

After an incubation period of 3–6 days, a prodrome characterized by low fever, malaise, and abdominal or respiratory symptoms precedes the mucocutaneous lesions by 12–24 hours. Adult cases of CVA6 in Europe and the United States have been associated with severe systemic symptoms and occasional onychomadesis (127).

Oral lesions typically appear first and are most common on the hard palate, tongue, and buccal mucosa. The lesions can vary in number from 1 to 10 and typically begin as macules that rapidly progress to 2- to 3-mm vesicles and then to shallow, yellow-gray painful ulcers with an erythematous halo.

Cutaneous vesicles appear concomitantly with or soon after the oral lesions and are most prevalent on the hands and feet, including the palms and soles, but can appear on the face, legs, and buttocks. These lesions can vary in number from a few to over 100. Cutaneous lesions also begin as erythematous macules, but are larger (3–7 mm) and develop into cloudy, white oval vesicles with a red halo (128). Both oral and cutaneous lesions are usually tender or painful and resolve in 5–10 days without treatment or scarring.

HFMD can cause neurologic manifestations that range from aseptic meningitis to acute flaccid paralysis and brainstem encephalitis, which can be associated with systemic features, such as severe pulmonary edema and shock, in many cases (129, 130).

Herpangina. Herpangina is a self-limiting, acute illness that characteristically affects the posterior oropharyngeal structures. Herpangina is usually caused by CVA2, CVA4, CVA5, CVA6, CVA8, or CVA10; however, less commonly, the syndrome can be caused by various group B coxsackieviruses, echoviruses, and nonspecific enteroviruses. Herpangina usually affects children from 1 to 7 years of age (131) and begins abruptly with a high fever, sore throat, dysphagia, anorexia, and malaise (132). Small, gray-white vesicles (less than 5 mm) surrounded by erythema appear on the posterior palate, uvula, and tonsils; the vesicles usually ulcerate. Systemic symptoms usually resolve within 4–5 days, and the ulcers heal spontaneously within 1 week.

TABLE 1 Common mucocutaneous manifestations of enteroviruses

Hand-foot-and-mouth disease
Herpangina
Macular rash
Maculopapular rash
Urticarial rash
Roseola-like lesions
Boston exanthem disease
Eruptive pseudoangiomatosis

TABLE 2 Associated manifestations of enteroviruses

Mild	Serious
Exanthem	Encephalitis
Enanthem	Meningitis
Fever	Neonatal sepsis
Pleurodynia	Myocarditis
Pharyngitis	Pericarditis
Croup	Hepatitis
	Acute paralysis

Diagnosis

Exanthematous enteroviral infections are usually diagnosed on the basis of clinical presentation. In both HFMD and herpangina, a mild leukocytosis (i.e., 10,000–15,000/mm^3) may be seen. If a specific diagnosis must be made, virus

isolation, type-specific serology, or reverse transcriptase (RT) PCR may be used to identify the responsible virus type. RT-PCR-based assays used to detect enterovirus serotypes are superior to viral culture for diagnosis of severe enterovirus infections, such as aseptic meningitis and encephalitis (133, 134). When a patient presents with vesicles, it is best to take samples from both throat swabs and vesicle swabs in attempts to isolate the virus or detect by RT-PCR. For those patients who do not have vesicles, it is best to take throat and rectal swabs (135).

Oral lesions of HFMD and herpangina can be distinguished from each other on the basis of the total clinical presentation (e.g., cutaneous and anterior oral lesions in HFMD) as well as serology. Oral lesions can sometimes be confused with aphthous stomatitis, which are larger and less uniform than the oral erosions in HFMD. More importantly, the mucous membrane ulcers should be differentiated from those associated with HSV and VZV. Cutaneous vesicles of HFMD should also be differentiated from HSV and VZV infections as well as from erythema multiforme, rubella, drug eruptions, and gonococcemia.

Therapy

There is no specific antiviral therapy for HFMD or herpangina; management is symptomatic. Fluid replacement and limited physical activity are strongly encouraged. Proper hygiene and avoiding contaminated food can help prevent viral infection. Breast feeding has also been found to reduce the number of enterovirus infections in infants and provides an overall protective effect (136).

Three vaccines against enterovirus 71 (EV71) have completed Phase III clinical trials with good safety and efficacy results (137). On December 3, 2015, the China Food and Drug Administration (CFDA) approved the first inactivated EV71 whole virus vaccine for preventing severe HFMD (138).

Measles Virus

Epidemiology

Measles, also known as rubeola, is a highly contagious childhood infection. Measles outbreaks typically occur in the late winter to early spring. Transmission is through respiratory droplets from sneezing and coughing. Infected persons are contagious for several days before signs and symptoms develop. Since the development of the measles vaccine in 1963, the incidence of measles has decreased by 98% in the United States (139), but 644 measles cases were reported in the United States in 2014, the highest number in the 21st century (due to noncompliance with recommended vaccinations). In many developing nations, however, measles is still highly prevalent; mortality rates range from 1% to 5% and can reach 30% in malnourished children and in refugee areas (140). Approximately 800,000 people worldwide still die from measles virus infection every year, with over half of these deaths in Africa (141).

Clinical Features

Infection is characterized by a prodromal phase of fever, coryza, cough, and conjunctivitis for 3–4 days. Splenomegaly and lymphadenopathy may also be noted. Cutaneous manifestations include pathognomonic Koplik's spots and a maculopapular morbilliform eruption. Koplik's spots, which appear several days before the onset of the rash, are characterized by clusters of blue-white spots on an erythematous base located on the buccal mucosa. The rash first appears on the forehead and behind the ears and then spreads inferiorly to the face, then the trunk and extremities, and finally to the palms and soles (Fig. 19). The macules and papules may coalesce, especially on the face. As the exanthema progresses, the systemic symptoms typically subside. The rash gradually fades to a yellow-tan color with faint desquamation, resolving entirely in 4–6 days. The measles rash is thought to be due to a hypersensitivity reaction. Children with defective cell-mediated immunity can develop measles without the characteristic rash, hindering clinical diagnosis.

Complications of measles virus infection include otitis media, pneumonia, encephalitis, diarrhea, purpura, and thrombocytopenia. In immunocompromised patients, both pneumonitis and encephalitis are more common. HIV-infected children have a higher rate of hospitalization from measles, a younger age of presentation, and a higher fatality rate (142). Atypical measles occurred in individuals previously given the formalin-inactivated (killed) measles vaccine that was in use in the United States from 1963 to 1968 (143). Coryza, conjunctivitis, and Koplik's spots are absent in atypical measles. Unlike in typical measles, the eruption spreads centripetally, usually beginning on the hands and feet. Initially, the exanthema consists of erythematous macules and papules which may progress to vesicular and petechial lesions (144). About 5% of measles-mumps-rubella vaccine recipients develop rash. The rash typically occurs 7–10 days after vaccination and lasts about 2 days.

Diagnosis

Diagnosis is usually made clinically, but serology using enzyme-linked immunosorbent assay (ELISA), complement fixation, neutralization, or hemagglutination inhibition tests

FIGURE 19 Measles in an infant.

FIGURE 20 Bacillary angiomatosis associated with *Bartonella quintana* in an human immunodeficiency virus (HIV)-positive man.

can be used for confirmation. In addition, virus isolation, antigen detection, and RT-PCR can be used. On cytological examination of secretions, multinucleated giant cells can be seen. Biopsy of the measles exanthem reveals hyaline necrosis of epithelial cells, formation of a serum exudate around superficial dermal vessels, and proliferation of endothelial cells, followed by a leukocytic infiltrate of the dermis and lymphocytic cuffing of vessels.

The differential diagnosis for measles includes other viral exanthems, such as rubella, roseola, and enterovirus, as well as drug eruptions, scarlet fever, Kawasaki disease, infectious mononucleosis, toxoplasmosis, and *Mycoplasma pneumoniae* infection (92).

Treatment

Measles is a self-limited disease in most patients, and treatment is largely supportive. Vitamin A deficiency is a risk factor for severe measles virus infection, and the WHO recommends vitamin A supplementation for all hospitalized patients in areas of high endemicity (145). There are currently no antiviral drugs specifically approved for the treatment of measles. Passive immunity via serum immunoglobulins may modify or prevent measles if administered within 6 days of exposure to the virus. Ribavirin may be beneficial in patients with severe complications of measles (146).

Rubella Virus

Epidemiology

Rubella, also known as German measles, is an acute viral illness typically seen in children, although all age groups are susceptible. Transmission is through airborne droplets and can occur up to 7 days before and after the onset of symptoms. Before the development of a vaccine in 1969, rubella was a worldwide disease with epidemics every 6–9 years, typically in the spring. Since the implementation of the vaccine, the incidence of rubella has decreased by 99% in the United States (92). However, with inadequate immunization programs in developing countries, rubella virus infection continues to be a significant health concern.

Clinical Features

Fifty percent of rubella virus infections are asymptomatic. When symptoms do occur, they usually begin with prodromal symptoms starting 14–21 days after infection. Prodromal symptoms are more prominent with increasing age and consist of low-grade fever, headache, conjunctivitis, upper respiratory tract symptoms, and sore throat. Lymphadenopathy is common, especially in the posterior auricular, suboccipital, and posterior cervical lymph nodes. Arthritis and splenomegaly can also occur, especially in adults. From 1 to 4 days after the initiation of the prodrome, an erythematous macular to papular rash appears. It begins on the face and spreads to the neck, trunk, and extremities within 24 hours. Lesions often coalesce to form a scarlatiniform eruption. The cutaneous manifestations typically resolve completely within 3 days without residual pigment changes. The rash appears simultaneously with a rise in antibody titers, suggesting that the exanthem may be due to the inflammatory effects of antibody–virus complexes rather than direct viral infection.

Rubella virus infection is typically a self-limited illness, although complications such as encephalitis, neuritis, orchitis, and thrombocytopenia can occur (147). Infection during pregnancy, especially in the first trimester, can lead to congenital malformations in approximately 50% of infected neonates. Neonates with congenital rubella can present with cataracts, deafness, congenital heart defects, intrauterine growth retardation, microcephaly, mental retardation, thrombocytopenia, hepatosplenomegaly, and encephalitis. Infection of the bone marrow produces the characteristic cutaneous findings of petechiae and ecchymoses (148).

Diagnosis

Diagnosis is typically made clinically, although it can be difficult to differentiate rubella from other viral exanthems. Increased numbers of atypical lymphocytes and plasma cells may be found in peripheral blood but are not diagnostic.

The mainstay of laboratory confirmation is the detection of rubella-specific IgM antibodies in serum samples. Alternative samples such as dried blood spots and oral fluids have been used for diagnosis by antibody detection. Oral fluids can also be used to detect viral RNA, and their use is becoming increasingly common because samples can be obtained safely and noninvasively, without the risks associated with blood collection. Thus, it improves patient compliance with specimen collection, as the procedure is simple and painless (149–151).

Diagnosis of congenital rubella can be made through viral culture, RT-PCR, or serology using samples from amniocentesis, cordocentesis, and chorionic villous sampling (147). During the first trimester, RT-PCR of a sample of amniotic fluid can provide a diagnosis of prenatal infection with rubella virus within 48 hours (152). After delivery, the detection of rubella-specific IgM antibodies can make the diagnosis of congenital rubella, as the maternally derived antibody is IgG.

The differential diagnosis for rubella includes other infectious exanthems, adverse drug eruptions, scarlet fever, and enterovirus infection (92). If arthritis is present, the differential diagnosis also includes acute rheumatic fever, rheumatoid arthritis, and erythema infectiosum (92). Congenital rubella syndrome may resemble other congenital infections due to toxoplasmosis and CMV.

Treatment

Rubella is typically a self-limited illness, and treatment is symptomatic. There are currently no antiviral medications specifically approved for rubella virus infection. Prevention is carried out with a live attenuated vaccine that is often administered as part of the measles, mumps, and rubella (MMR) vaccine or the recently developed measles, mumps, rubella, and varicella (MMRV) vaccine. Since the implementation of the vaccine, rubella is no longer considered endemic in the United States (153).

Hepatitis C

Epidemiology

It is estimated that approximately 3% of the world's population are living with chronic hepatitis and that about 3–4 million people are infected per year (154, 155). In the United States, approximately 2.7 million persons have chronic HCV infection. The infection is most prevalent among those born during 1945–1965. The incidence of HCV has decreased from approximately 230,000 per year in 1980 to the current level of approximately 30,000 cases per year (155).

The predominant risk factor for HCV transmission is injection drug use. Other risk factors include blood transfusion (although rare since routine testing of the blood supply for HCV began in 1990), needle stick, sex, and nosocomial transmission. HCV infection becomes chronic in approximately 75–85% of cases (155).

Clinical Manifestations

Multiple dermatologic diseases may be associated with hepatitis C virus (HCV) infection including porphyria cutanea tarda (PCT), lichen planus, leukocytoclastic vasculitis, mixed cryoglobulinemia, and necrolytic acral erythema (156).

PCT is a disease caused by altered activity of the enzyme uroporphyrinogen decarboxylase (UROD), which leads to buildup of uroporphyrinogen in the blood and urine (157). A systematic review including 50 studies and 2167 patients with PCT found an overall prevalence of HCV of 50% (158). The exact mechanism by which HCV infection increases PCT risk is unknown.

The characteristic skin findings of PCT are photosensitivity and skin fragility, with which exposure to the sun and/or minor trauma can lead to skin erythema and the development of vesicles and bullae that may become hemorrhagic. Hyperpigmentation, hypopigmentation, hirsutism, and sclerodermatous changes may occur over time. Precipitating factors (sun exposure, polyhalogenated hydrocarbons, alcohol, estrogens, and iron overload) are thought to be necessary to provoke PCT. The diagnosis of PCT is suspected clinically and is confirmed by the presence of markedly elevated urine uroporphyrin levels. Management of PCT in patients with HCV infection includes avoiding precipitating factors and treating HCV infection. Improvement of PCT during HCV treatment has been described (159).

Leukocytoclastic vasculitis may occur in conjunction with essential mixed cryoglobulinemia, presenting with palpable purpura and petechiae that usually involve the lower extremities. Skin biopsy demonstrates cutaneous vasculitis with dermal blood vessel destruction and a neutrophilic infiltration in and around the vessel wall.

Lichen planus (LP) is characterized by flat-topped, violaceous, pruritic papules involving the skin, oral mucosa, scalp, nails, and genitalia. LP can be seen in patients in a variety of liver diseases including primary biliary cirrhosis and chronic active hepatitis or cirrhosis of unknown cause (160–163).

Anti-hepatitis C virus (HCV) antibodies are present in 10–40% of these patients, although a causal association is uncertain. There are reports of the development or exacerbation of LP during chronic HCV treatment with IFN. In one case report, the lesions improved when IFN was stopped (164).

Necrolytic acral erythema is a pruritic, psoriasis-like skin disease characterized by sharply marginated, erythematous to hyperpigmented plaques with variable scale and erosion on the lower extremities. In a series of 30 patients who presented with the disorder, all were found to have antibodies to HCV (165). Biopsy specimens showed psoriasiform changes, keratinocyte necrosis, and papillomatosis. Topical and systemic corticosteroids have a variable benefits. Other reports have confirmed improvement with IFN-α and also suggest a benefit from oral zinc sulfate (166–168).

Diagnosis

Initial diagnostic evaluation for chronic HCV begins with an antibody test (anti-HCV). A reactive antibody test should be followed by HCV RNA to confirm viremia. The average time to detection of anti-HCV after exposure is 4–10 weeks after infection. Anti-HCV can be detected in >97% of patients by 6 months after exposure.

Treatment

Treatment selection varies by genotype and other patient factors (see Chapter 53: Hepatitis C Virus Authors: Yaron Rotman, T. Jake Liang). Until recently, the mainstay of treatment for chronic HCV infection has been pegylated IRN (peg-IFN) and ribavirin, with possible addition of protease inhibitors (boceprevir and telaprevir) for HCV genotype 1 infection. This treatment for 24–48 weeks resulted in a cure in 50–80% of patients (higher in patients with HCV genotypes 2 and 3 than genotype 1) (169). In late 2013, the FDA approved two new direct-acting antiviral drugs, sofosbuvir (nucleotide analogue inhibitor of HCV NS5B polymerase enzyme) and simeprevir (protease inhibitor) to treat chronic HCV infection. Clinical trials have shown that these new medications achieve sustained virological response (SVR) in 80–95% of patients after 12–24 weeks of treatment (169).

Some of the cutaneous manifestations that may respond to treatment for HCV include PCT, leukocytoclastic vasculitis, mixed cryoglobulinemia, and necrolytic acral erythema.

Human Immunodeficiency Virus

Epidemiology

HIV type 1 (HIV-1) and other retroviruses can cause significant mucocutaneous manifestations. These manifestations are often the signs and symptoms that first raise suspicion for HIV infection and prompt serologic testing for it (170). Similarly, a variety of mucocutaneous manifestations may serve as clinical markers of progression from asymptomatic HIV infection to full-blown AIDS (171).

Transmission of HIV is primarily via sexual contact with an infected person, significant exposure to infected blood or blood products, or perinatally from an infected mother to her child. After sufficient contact with HIV, the virus infects $CD4^+$ T lymphocytes by binding to its receptor, the CD4 molecule, and coreceptors (CCR5 or CXCR4). Other $CD4^+$ cells, such as monocytes and macrophages, are also infected by HIV and help to spread the virus to susceptible cells in the brain, lymph nodes, skin, lungs, and gastrointestinal tract. HIV pathophysiology involves killing $CD4^+$ cells as well as the induction of an immune response and cytokine production.

Clinical Features

Patients with acute primary HIV infection often become symptomatic 3–6 weeks after exposure and manifest fever, mononucleosis-like symptoms, and a characteristic erythematous, maculopapular exanthem appearing on the trunk and extremities. The exanthem and symptoms of the

TABLE 3 Dermatologic findings of opportunistic diseases in HIV-infected patients

Organism(s)	Dermatologic findings
Viruses	
Molluscum contagiosum (MC) virus	Dome-shaped, flesh-colored papules with a central umbilication; larger, coalescent, and persistent lesions occur in HIV-infected patients; lesions may be widespread and atypical; observed in unusual sites such as the face, neck, and scalp; unusual forms include solitary, endophytic, aggregated, inflamed, and giant MCs (73)
Herpes simplex virus (HSV)	Clusters of vesicles that may rupture, crust, and form multiple small or large confluent painful ulcers; recurrent oral and anogenital HSV may lead to chronic ulcerations in HIV-infected patients (127)
Herpes zoster virus (shingles)	Dermatomal eruption of vesicles that arise in clusters from a red base that either umbilicate or rupture before forming crusts; in HIV-infected patients, the eruption may also be multidermatomal, recurrent, ulcerative, and widely disseminated with systemic involvement (29)
Varicella-zoster virus (VZV; chickenpox)	HIV-infected patients often have chronic infections that begin as vesicles and progress to necrotic, nonhealing ulcers (77)
Human papillomavirus (HPV; warts)	Flesh-colored papules that evolve into dome-shaped, gray-to-brown, hyperkeratotic discrete, and rough papules, often with black dots on the surface; HIV-infected patients can have severe, widespread, and chronic warts, which may arise on mucosal surfaces, the face, perianal region, and the female genital tract; HPV is associated with cervical cancer in women (81) and anal cancer in both sexes
Epstein-Barr virus (EBV; oral hairy leukoplakia)	White plaques with hair-like projections localized on the lateral aspect of the tongue (113)
Cytomegalovirus (CMV)	Persistent perineal ulcers are the most common presentation; ulcers may be coinfected with HSV; also associated with nonspecific cutaneous lesions such as verrucous or purpuric papules, vesicles, morbilliform eruptions, and hyperpigmented indurated plaques (29, 81, 91)
Bacteria	
Staphylococcus aureus	Primary infections include impetigo, folliculitis, furuncles, carbuncles, abscesses, and necrotizing fasciitis; recurrent infections are common due to increased prevalence of nasal and perineal colonization in HIV-infected patients (63, 177)
Pseudomonas aeruginosa	Ecthyma gangrenosum, infection of catheter sites, and secondary infection of underlying disorders such as Kaposi's sarcoma in advanced HIV disease (81)
Bartonella spp. (bacillary angiomatosis)	Bacillary angiomatosis is characterized by red-to-purple papules, nodules, or plaques resembling Kaposi's sarcoma (Fig. 20); any site except the palms, soles, and oral cavity may be involved; hematogenous or lymphatic dissemination to bone marrow and other lymphoid organs may occur (175)
Mycobacterium tuberculosis	Cutaneous tuberculosis is rare; multifocal lupus vulgaris, tuberculous gummata, orofacial tuberculosis, scrofuloderma, and miliary abscesses may be seen (81)
Mycobacterium avium-intracellulare complex	Cutaneous manifestations are extremely rare, and some reports describe scaling plaques, crusted ulcers, ecthyma-like lesions, verrucous ulcers, inflammatory nodules, pustular lesions, and draining sinuses (81)
Treponema pallidum (syphilis)	Although the classic papulosquamous secondary lesions are often seen, unusual presentations may be observed in HIV-infected patients, including rapidly progressing noduloulcerative forms, papular eruptions that mimic MC, and lues maligna (110)
Fungus or infection	
Candida (candidiasis)	Generally causes mucosal disease (cutaneous, oropharyngeal, vulvovaginal, and esophageal); recurrent and persistent mucocutaneous candidiasis is common in HIV-infected patients; manifests as whitish, curd-like exudates on the dorsal or buccal mucosa that are easily scraped away; recurrent vulvovaginal candidiasis presents with creamy-white vaginal discharge, with itching and burning pain; the vaginal mucosa is inflamed, and pseudomembranous plaques are often seen (63, 105)

Tinea versicolor (dermatophytosis)	Numerous small, circular, white, scaling papules on the upper trunk; may involve the upper arms, neck, and abdomen; in HIV-infected patients, cutaneous involvement is often more atypical in appearance, widespread, and resistant to therapy (58)
Cryptococcus neoformans (cutaneous cryptococcosis)	Translucent, dome-shaped, umbilicated lesions resembling MC on the head, face, and neck; cellulitis, ulcers, papules, plaques, and pustules are other presentations (81)
Histoplasma capsulatum (cutaneous histoplasmosis)	Mucocutaneous erosions, oral ulcerations, disseminated and erythematous macules and papules, cellulitis-like eruptions, MC-like lesions (108)
Coccidioides immitis (coccidioidomycosis)	Begins as papules and evolving to pustules, plaques, or nodules with minimal surrounding erythema; hemorrhagic papules or nodules; lesions may resemble MC (81)
Sporothrix schenckii (sporotrichosis)	Hematogenous dissemination to the skin may manifest as papules to nodules that become eroded, ulcerated, crusted, or hyperkeratotic, usually sparing the palms, soles, and oral mucosa (79)
Penicillium marneffei (penicilliosis)	Most common skin lesions are umbilicated papules resembling MC, occurring most frequently on the face, ears, upper trunk, and arms (81)
Aspergillus spp.	Necrotic papulonodules; subcutaneous nodules (138)
Parasites	
Leishmania donovani (leishmaniasis)	Papular, maculopapular or nodular lesions; typically ulcerated nodules on the extremities; in atypical presentations, the lesions are disseminated (99)
Acanthamoeba castellani (acanthamebiasis)	Dissemination to the skin is common in AIDS; necrotic nodules and painful ulcerations of the trunk and extremities develop in HIV-infected patients (29, 146)
Toxoplasma gondii (toxoplasmosis)	Cutaneous involvement is rare; manifests as an eruption of macules, papules, or vesicles involving the trunk and extremities (81)
Pneumocystis carinii	Disseminated infection may appear as MC-like papules, bluish cellulitic plaques, and deeply seated abscesses in the external ear or nares (81)
Malignancies	
Kaposi's sarcoma	Purple patches on the distal lower extremities that progress proximally and become multifocal; individual lesions darken and thicken, eventually becoming brown and verrucous; lesions in HIV-infected patients have a predilection for the face, torso, and oral mucosa (7)
Non-Hodgkin's lymphoma	Non-Hodgkin's lymphoma tends to be more progressive and aggressive (88); pink-to-purple papules are usually seen when the skin is affected; the lesions often ulcerate and sometimes stimulate panniculitis (176); younger age of onset, more advanced stages, and extranodal site involvement at presentation, in particular the central nervous system, intestine, and skin, are found in HIV-infected patients (87)
Squamous and basal cell carcinomas	In HIV infection, these tumors appear earlier and more often on unexposed sites such as the trunk and extremities; metastases of basal cell carcinoma have been recorded (103, 152)
Malignant melanoma	Appears to be more aggressive, with shorter disease-free periods and lower overall survival rates in patients with melanoma and HIV than in patients with melanoma without HIV (2)

TABLE 4 Antiretroviral therapy cutaneous adverse effects

Adverse effect	Nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs)	Nonnucleoside reverse transcriptase inhibitors (NNRTIs)	Protease inhibitors (PIs)	Integrase strand transfer inhibitors (INSTIs)	Entry inhibitor
Rash	Emtricitabine: hyperpigmentation	All NNRTIs	Atazanavir, darunavir, fosamprenavir, lopinavir/ritonavir, tipranavir	Raltegravir, elvitegravir	Maraviroc
Hypersensitivity reaction (HSR)	Abacavir: median onset 9 days; 90% of reactions occur within first 6 weeks of treatment	Nevirapine: hypersensitivity syndrome of hepatotoxicity and rash that may be accompanied by fever, general malaise, fatigue, myalgias, arthralgias, blisters, oral lesions, conjunctivitis, facial edema, eosinophilia, renal dysfunction, granulocytopenia, or lymphadenopathy	N/A	Raltegravir: HSR reported when raltegravir given in combination with other drugs known to cause HSR	Maraviroc: reported as part of a syndrome related to hepatotoxicity

acute illness generally resolve spontaneously within 2 weeks (167). Biopsy material from the exanthem associated with primary HIV infection usually demonstrates nonspecific changes, such as a superficial perivascular and perifollicular mononuclear cell infiltrate predominantly composed of $CD4^+$ cells. The production of anti-HIV antibodies at detectable levels usually requires several weeks and in some cases may follow infection by more than 1 year.

As the $CD4^+$ T cells decline and the disease progresses from asymptomatic HIV infection to AIDS, over 90% of patients will develop mucocutaneous manifestations (168). These may be a direct consequence of the primary HIV-1 infection or the result of secondary infectious, neoplastic, inflammatory, or other processes. The dermatologic findings of opportunistic HIV/AIDS-related pathogens include viral, bacterial, fungal, and parasitic infections, as well as malignancies (Table 3). Inflammatory diseases (e.g., psoriasis and Reiter's disease), vascular diseases, hypersensitivities to drugs, insect bites, and ultraviolet light, pruritus, xerosis, ichthyosis, and seborrheic dermatitis are all common nonneoplastic/noninfectious etiologies.

Mucocutaneous manifestations of acute HIV infection are nonspecific and can resemble those of a variety of infectious diseases, including enterovirus infection, infectious mononucleosis, secondary syphilis, acute infection with hepatitis A or B viruses, roseola, and toxoplasmosis (172, 173). The papulosquamous eruption of HIV most closely mirrors that of secondary syphilis, but drug eruptions are also included in the differential diagnosis. Because a wide variety of skin problems develop in persons with advanced HIV disease, the differential diagnosis usually expands into a myriad of possibilities that often can be differentiated one from another only via a skin biopsy.

Diagnosis

ELISA is used to screen for HIV infection, and the Western blot assay is used to confirm the diagnosis. Although seroconversion does not occur until approximately 6 weeks after the acute illness, viremia can be detected approximately 10 days after infection (174). The presence of HIV may be detected by PCR or isolation of virus from the blood or demonstration of HIV p24 antigenemia. Semiquantification of HIV RNA in the serum, which is useful in assessing the response to antiretroviral therapy, can also be done using PCR. Disease progression is also accompanied by a marked decline in $CD4^+$ cells, an increase in $CD8^+$ cells, and an inverted CD4/CD8 cell ratio.

Treatment

There is an expanding number of drugs that have been approved for treatment of HIV infection (see Chapters 11 and 33). Multiple antiretroviral therapy causes rash and hypersensitivity reaction (HSR) (Table 4) (178).

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Viral Hemorrhagic Fevers

GAIL CARSON, MIKE BRAY, AND CATHY ROTH

9

The term viral hemorrhagic fever (VHF) designates a syndrome resulting from infection with any of at least 30 different RNA viruses from four different taxonomic families (Table 1). Although they differ in certain features, all types of VHF are characterized by fever and malaise, a fall in blood pressure that can lead to shock, the development of coagulation defects that can result in bleeding, and for many VHF agents, high mortality. With the exception of dengue virus, which is maintained among human populations by mosquito transmission, all of the VHF agents persist in nature through cycles of infection in animals. In the past, therefore, the geographic range of each disease reflected that of the reservoir species. Human illness is an accidental event resulting from contact with an infected animal or its excretions or the bite of an infected arthropod. Subsequent human-to-human transmission through contact with infectious blood or secretions occurs with multiple haemorrhagic fever (HF) viruses and can cause devastating nosocomial outbreaks. Pathogenesis in humans, in most instances, only indirectly reflects the mechanisms by which the causative agent replicates in its reservoir host, but high levels of viremia are typical. Treatment is supportive for most VHFs, but progress is being made gradually in developing specific therapeutics. Vaccines are widely available for yellow fever, and recent studies indicate that effective dengue and Ebola virus vaccines are possible.

HISTORICAL PERSPECTIVES

The various types of VHF have presumably occurred for millennia, whenever humans have come into contact with reservoir animals or been bitten by infected arthropods. The first to be recognized by the European medical community was yellow fever (YF), which was encountered by early travelers to sub-Saharan Africa and was transferred to the New World through the slave trade. Its frequently fatal outcome was long attributed to the severe hepatic damage and jaundice that gave the disease its name, but it was eventually realized that gastrointestinal hemorrhage and compromised renal function resulting from hypovolemic shock were more common causes of death. A correct appreciation of the role of diminished intravascular volume in this disease did not emerge until clinical tools for blood pressure measurement were developed in the 1920s, when urban YF had been largely suppressed by mosquito control efforts. Recent studies have in fact shown that host inflammatory responses are as important in the pathogenesis of severe YF as in other types of VHF (1, 2).

Other types of VHF began to be identified in the early 1900s, when severe hantaviral infection that is now termed HF with renal syndrome (HFRS) was described in Siberia, and a milder form, nephropathia epidemica, was recognized in Scandinavia (3, 4, 5). However, it was not until several thousands of cases of HFRS occurred among United Nations troops in the Korean War that VHF was brought forcefully to the attention of western medicine. Over the ensuing five decades, a number of "new" types of VHF have been described and their causative agents have been isolated. The Old World arenavirus Lassa fever virus and the New World agent Machupo virus were both characterized during investigations of disease outbreaks in the 1960s. Marburg virus was discovered in 1967 as a result of the inadvertent importation of infected monkeys from Uganda to Europe, while the other filovirus genus, Ebola virus, came to attention when its Zaire and Sudan species caused large epidemics in Africa in 1976. Rift Valley fever (RVF), first recognized in the 1930s, caused a massive mosquito-borne outbreak in Egypt in 1977. The list of arenaviruses causing a VHF syndrome has also continued to grow with the recognition of fatal human infections caused by Whitewater Arroyo virus in California (6). Lujo, a "new" arenavirus, emerged in Zambia in 2008, and was exported to South Africa, where it caused a small but deadly outbreak with a case/fatality rate of 80% (7). Its natural reservoir has never been identified, and there have been no further outbreaks in the intervening years, but its emergence serves as a reminder that there is no reason to believe that the list of HF viruses is now complete. That is exactly what happened in 2009 in the Democratic Republic of Congo with the discovery of a novel rhabdovirus, called Bas-Congo virus, as a cause of an outbreak including haemorrhagic presentation and fatalities and with emergence of tick-borne phleboviruses causing Severe Fever with Thrombocytopaenia Syndrome (SFTS) in Asia and cases of a similar illness due to Heartland virus in the United States (8, 9, 10).

The West African outbreak of Ebola virus disease (EVD) of 2013 to 2016, in which the estimated number of cases was beyond all previous experience and expectations, has changed our understanding of VHFs and in particular EVD

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TABLE 1 Some epidemiological features of the principal types of VHF

Virus family	Disease	Virus	Geographic distribution	Reservoir host	Patients[a] and areas affected; seasonal pattern
Arenaviridae	Argentine HF	Junin virus	North-central Argentina	Mouse (*Calomys musculinus*)	M; corn harvest; March–June
	Bolivian HF	Machupo virus	Northeastern Bolivia	Mouse (*Calomys callosus*)	All ages, both sexes; villages; February–July
	Chapare	Chapare virus	Bolivia	Unknown rodent	
	Venezuelan HF	Guanarito virus	Central Venezuela	Mouse (*Zygodontomys brevicauda*)	All ages, M = F; houses, gardens; no seasonality
	Brazilian HF	Sabia virus	Unknown	Unknown rodent	Two cases in the Sao Paulo state and two in lab workers
	Lassa fever	Lassa virus	West Africa	Mouse (*Mastomys natalensis*)	All ages, both sexes; villages; no seasonality
	Lujo	Lujo virus	Zambia	Unknown rodent	Only one small outbreak; difficult to comment on any patterns or at risk groups
Bunyaviridae	CCHF	CCHF virus	Africa, central to east Europe, Middle East to west China	Livestock, crows, hares, *Hyalomma* ticks	Adults, M > F; cattle, pasture contact; summer
	RVF	RVF virus	Africa	Livestock, several mosquito genera	All ages, M > F; late summer; arthropods
	HFRS	Hantaan, Seoul, and Puumula viruses	Northern Asia and Europe, including the Balkans and Scandinavia	Mice and rats (*Apodemus*, *Rattus*, *Clethrionomys*)	Mostly adults, M > F; rodent excreta; fall-winter
	Hantavirus cardiopulmonary syndrome	Sin Nombre, Andes, many others	North, Central, and South America	Mice (*Peromyscus* sp., *Sigmodon hispidus*, ?others)	Adults, M = F; rodent excreta; late spring-summer peak
Flaviviridae	YF	YF virus	Tropical Africa, Amazon basin	Primates, including humans; tree hole mosquitoes	M > F, all ages; arthropod contact; dry season
	Dengue HF/shock syndrome	Dengue virus types 1–4	Southeast Asia, Caribbean, South and Central America	*Aedes aegypti* > *Aedes albopictus*	Children < 12 yr; peak in late rainy, early dry seasons
	Kyasanur Forest disease	Kyasanur Forest virus	Karnatake State (India)	Monkeys, birds, livestock, ixodid ticks	Adults, M > F; tick contact; summer-fall, dry season
	Omsk HF	Omsk virus	Western Siberia	Vole (*Arvicola terrestris*), ixodid ticks	Adult males; muskrat hunt; winter
Filoviridae	Marburg and Ebola HF	Marburg and Ebola viruses	Sub-Saharan Africa & West Africa	Fruit bat species likely reservoir	Mainly adults, M = F; sporadic; late summer

[a]M, male; F, female.

in many ways (see chapter 42). These include advances in understanding the molecular epidemiology and biology of the virus, investigating new drugs and vaccines, defining standards of care and their delivery, and highlighting the impacts on survivors, including viral persistence and associated sequelae.However, many unanswered questions remain concerning fundamental aspects of EVD pathogenesis and clinical management. Despite unprecedented efforts, few therapeutic and vaccine trials came to completion for various reasons, including inability to meet enrolment targets due to declining case numbers as the epidemic waned. All of these experiences have led to a global consensus that new approaches must accelerate the necessary research and product development (diagnostics, drugs, and vaccines) to prevent devastating epidemics of all future VHF diseases. In 2015 the World Health Organization (WHO) launched its R&D Blueprint in response to the call for greater coordination and leadership for research and development (R&D) (11). The R&D Blueprint is a global strategy and preparedness plan to ensure that targeted R&D can strengthen the emergency response by bringing medical technologies to patients during epidemics. The Blueprint aims to reduce the time between the detection of a disease likely to cause a public health emergency and the availability of effective tests, vaccines, and medicines that can be used to save lives and avert crisis. Of note, one-half of the infections on the list of priority diseases for R&D are VHFs.

CAUSATIVE AGENTS

The principal HF viruses belong to four different families, the *Arenaviridae* (chapter 45), *Bunyaviridae* (chapter 44), *Filoviridae* (chapter 42), and *Flaviviridae* (chapter 53). All are enveloped viruses with single-stranded RNA genomes. The flavivirus genome consists of one strand of positive-sense RNA. Viruses in the other three families have negative-sense genomes, which consist of a single strand in the case of the filoviruses, two separate segments for the arenaviruses, and three for the bunyaviruses. These agents differ widely in cellular replication strategies, natural hosts and transmission cycles, geographic distribution, routes of transmission to humans, and disease pathogenesis (Table 1). This chapter provides a general survey of VHFs, comparing individual diseases to each other and to other types of human illness. Additional information on individual viruses and the diseases they cause can be found in the pathogen-specific chapters of this book, as well as those on viral infections that may uncommonly be associated with hemorrhagic manifestations (e.g., measles, fulminant hepatitis, and historically smallpox).

EPIDEMIOLOGY

For a zoonotic virus to cause illness, appropriate cell surface receptors and intracellular cofactors must be present to permit its replication in human cells, and innate defenses must fail sufficiently to permit its intrahost spread. VHF represents the extreme end of the spectrum of possible outcomes of cross-species virus transfer, in which an agent replicates so well and overcomes or interacts with host immune defenses to cause a severe inflammatory syndrome.

Reservoirs have been identified for nearly all of the HF agents (Table 1). In several cases, the natural reservoir is a species of rodent, probably because these animals' large numbers and high population density favor the continuous circulation of viruses. Arenaviruses, which are split into "Old World" (Africa) and "New World" (the Americas) complexes on the basis of phylogenetic and geographical distinctions, all have rodent species as the natural reservoir. Old and New World primates are reservoirs for sylvatic YF, and a variety of species are involved in the circulation of Crimean-Congo HF (CCHF) virus. The reservoir host for the filoviruses is believed to be various bat species (see chapter 42). Other animals may be infected and act as intermediaries in transmission to man.

The transmission of a virus from animals to humans can occur through direct physical contact, exposure to virus-containing excretions, or the bite of an infected mosquito or tick. The identity of the host and the mode of transmission strongly influence the pattern of human disease. For example, CCHF, which is transmitted by tick bite or direct contact with the tissues of an infected animal during slaughter, tends to occur as sporadic single cases or small clusters. By contrast, mosquito-borne agents such as YF virus and dengue virus can be carried from person to person by the vector, or, as in the case of RVF, animal to person and person to person by the vector to yield explosive epidemics. The modern health care setting offers opportunities to amplify sporadic cases into outbreaks if appropriate infection prevention and control practices are not observed; health care workers are often among the earliest casualties.

The arenaviruses and hantaviruses provide interesting examples of coevolution, in which each agent has a single rodent species as its primary host (12). Animals that harbor arenaviruses display partial immune "tolerance," permitting chronic viremia and viruria; the latter probably represents the major source of environmental exposure. The epidemiological pattern of arenaviral HF is determined by the intersection of rodent ecology and human activities. In Argentina and Venezuela, animal reservoirs are found in or adjacent to cultivars, placing adult males who harvest corn in the fall at greatest risk of infection. In contrast, because the reservoir rodents invade dwellings and gardens, Bolivian HF and Lassa fever are largely acquired in or near houses, and persons of both sexes and all ages are at risk. Lassa fever is a truly endemic disease, because the *Mastomys* reservoir breeds year round, and a nearly constant fraction of animals are chronically infected. Hantaviruses also cause chronic infection in their rodent hosts, which excrete virus in saliva and feces for short periods and in urine for many months. Human infection is most often associated with agriculture, mining, or military activity. Nephropathia epidemica has a well-marked cyclic activity, in which rodent population density and prevalence of infection correlate with transmission to humans.

The epidemiology of arthropod-borne VHF reflects the biology of viral infection in the mosquito or tick vector. Both vectors acquire the virus through blood feeding, indicating that viremia occurs in the animal reservoir. Mosquitoes competent to transmit flaviviruses become chronically infected, and there may be transovarial transmission to subsequent generations of mosquitoes, allowing for persistence and recrudescence when the seasonal or other environmental conditions permit. Multiple human infections are often the result of interrupted blood feeding and movement to a second host. Among the various types of VHF, CCHF, Kyasanur Forest disease, Alkhurma HF, SFTS, and Omsk HF are transmitted by ticks. "Vertical" (transstadial and transovarial) transmission is an important feature of their natural history. Ticks use the blood of birds and mammals primarily as an energy source for the next stage of their life cycle. Because far less than 100% of eggs from an adult female are infected, vertebrate viremia is also important for tick-borne transmission of virus to humans.

Dengue fever is the exception to the rule that the HF viruses are zoonotic in nature. The disease may at one time have been confined to a small region of the tropics and maintained through infection of wild primates, in a manner similar to YF. However, the successful adaptation of the virus to person-to-person transmission by mosquitoes, combined with a vast increase in the human population in tropical regions and a failure of mosquito control efforts, has permitted the agent to disperse widely and evolve into four distinct serotypes (13). As discussed below, the circulation of more than one serotype in the same geographic region sets the stage for the occurrence of secondary infections, in which non-neutralizing antibodies are believed to enhance virus uptake into cells, causing intense inflammation and increased vascular permeability (dengue HF/shock syndrome). Even though only a small fraction of cases result in HF, because of its global distribution, dengue virus is the most important cause of VHF.

Once an individual becomes ill with a VHF, there is great variation in the potential for further human-to-human transmission, because the pathogenesis of the diseases and sites of viral replication within the infected human host are so diverse. A number of diseases, including RVF and dengue, rarely spread directly from person to person and thus pose little threat to medical personnel. However, some of the most virulent agents, including Ebola, Marburg, Lassa, Lujo, and CCHF viruses, cause prolonged high titer viremia, and can therefore be spread through direct contact with blood and other body fluids and tissues. Hospital-based outbreaks have occurred when case detection has been slow, staff have been overwhelmed by the workload, or appropriate infection prevention and control measures have not been observed. In low-technology health care settings in developing countries, the necessary disinfection and personal protective equipment required to implement the appropriate measures are often not available (14).

The extent to which asymptomatic or mildly symptomatic individuals are involved in onward transmission of infection and extension of outbreaks varies among the VHFs, and has not been well explored, even for those diseases where mildly symptomatic infection is not uncommon, e.g., Lassa fever. Asymptomatic infection and continuing human-to-human transmission had always been assumed to occur extremely rarely, if at all, in filovirus outbreaks. Sexual transmission in Ebola and Marburg was recognized in the past, but more evidence has been collected during the recent West African epidemic, where on several occasions it has instigated new cases and clusters long after the apparent end of virus circulation in specific communities. In one survivor, semen was positive for viral RNA 284 days after symptom onset (15). The full extent of "sanctuary sites," and duration of the survival of viable virus in those sites after an individual's apparent recovery from illness, have not yet been determined, but studies continue to explore this in survivors of the EVD epidemic in West Africa. There are anecdotal reports of CCHF being transmitted sexually, and therefore this route of transmission should be considered in subsequent outbreaks. Sexual transmission may have a role in some other VHFs, including Lassa, Junin, and Machupo; better studies are required to determine the frequency and importance of sexual transmission in the propagation of outbreaks of these and other VHFs. During VHF outbreaks, transmission by infectious blood products is also a possibility.

PATHOGENESIS

The transmission of HF viruses from animals to humans is generally unidirectional: infected patients do not often appear to serve as a source of infection for the reservoir or other intermediary host. Because human infections are "dead-end" events from the point of view of virus evolution, the pathogenesis of VHF does not represent the outcome of viral adaptation or "survival strategy," but simply reflects the fortuitous ability of an animal virus to replicate efficiently in human cells. In contrast to diseases such as viral hepatitis or encephalitis, VHFs do not generally localize to one organ or organ system. However, the persistence of pathogens like Ebola virus in semen and the associated possibility of onward transmission raise new concerns. In general, HF viruses replicate primarily in monocytes, macrophages, and dendritic cells. The fact that these cells, which normally serve as the first line of defense against microbial invaders, are the principal sites of viral replication goes far to explain the ability of these pathogens to cause rapidly overwhelming infection. However, HF viruses differ in their abilities to infect other types of cells. At one end of the spectrum, dengue virus principally infects only these cell types, without causing their death. At the other extreme, Ebola and Marburg viruses show a very broad tissue tropism. Material released from dying cells is itself a stimulus for inflammation, contributing further to the fulminant systemic illness. Most HF viruses produce a degree of tissue damage intermediate between the minimal injury of dengue fever and the massive destruction caused by the filoviruses, with the liver as the principal target. Hepatic involvement probably begins with the spread of virus through the bloodstream to fixed macrophages (Kupffer cells) in sinusoids, from which infection then extends to parenchymal cells. As noted, hepatic injury is a prominent feature of YF, causing the jaundice that gave the disease its name, and is seen in some cases of RVF and dengue. It also occurs in CCHF and some other infections, but without producing the high levels of bilirubin that lead to jaundice.

For many reasons, studying the pathogenesis of the VHFs is challenging due to biosafety requirements, limitations of animal models in recapitulating human disease, and the challenge of obtaining samples during outbreaks. One of the most studied VHFs is EVD, yet many questions remain unanswered regarding its pathogenesis and multiple factors likely contribute (see chapter 42). The current data in some ways support the hypothesis presented by Bray and Mahanty in 2004 following the Ugandan outbreak of Ebola Sudan that the multiorgan syndrome induced by Ebola virus (EBOV) is similar to that of septic shock (16). With its broad cell tropism, Ebola virus infects monocytes, macrophages, dendritic cells, endothelial cells, fibroblasts, hepatocytes, adrenal cortical cells, and several types of epithelial cells (17, 18, 19, 20, 21). An early, well-regulated inflammatory response has been associated with recovery, whereas a dysregulated proinflammatory response has been described in fatal EVD (22). Depletion of lymphoid cells including apoptosis of these cells may partly explain the lymphopenia and associated failure of adaptive immune responses in filoviral infections and probably in other types of severe HF (23, 24).

The VHF agents differ in their interactions with the immune system, but most share a common feature of the inhibition of the type 1 interferon response. A number of HF agents, including filoviruses, RVF virus, dengue virus, and some arenaviruses, have been shown to block interferon responses through a variety of mechanisms (25, 26, 27). The importance of the interferon system to the control of viral dissemination helps to explain why all of the HF agents are RNA viruses. Because the double-stranded RNA molecules that are generated in the course of their transcription and genome replication are a strong stimulus for type I interferon

responses, each RNA virus must evolve ways of evading or suppressing interferon responses in its host, to the extent needed to ensure its own continued survival. The outcome of human infection with a novel virus will therefore depend in part on the extent to which the agent blocks human interferon responses. The HF viruses may constitute that small subset of RNA viruses that suppress human interferon responses so effectively that they cause rapidly overwhelming disease. The most severe diseases, such as fatal Ebola and Marburg HF, are also characterized by a failure of humoral and cellular immune responses. Others like RRVF and HFRS proceed despite the host's adaptive immune response. Most RVF cases are acute, self-limited febrile episodes in which viremia may be very high at onset but disappears within 3 to 5 days, by which time virus-specific antibodies are detectable. However, about 1 to 2% of patients continue to have virus in the blood, respiratory secretions, and spinal fluid, even in the presence of antibodies, and progress to fulminant VHF with hepatitis, jaundice, and hemorrhage. Uncontrolled viral replication in these RVF patients therefore resembles that seen in filoviral HF, but it appears to result from a defective immune response in certain patients rather than the increased virulence of a viral strain. In another disease variant, some RVF patients appear to be stable or improving after 5 to 15 days of illness and then develop an apparently immune-mediated meningoencephalitis or retinal vasculitis. The neurologic disease has been temporally associated with the greatest production of antiviral antibodies seen in any of the forms of RVF. Clarification of the mechanisms of RVFV invasion into the CSF requires further study. Virtually all patients with hantavirus infections have circulating virus-specific immunoglobulin M, immunoglobulin G, or both at the time of diagnosis. Viral antigens are detectable on the surfaces of capillary endothelial cells, in the kidneys in HFRS (28). The development of HFRS and its resolution take much longer, indicating that tissue damage is greater, needs more time for repair, and may be partly the result of viral destruction of cells or the effects of immune viral complexes.

Dengue HF has a unique pathogenesis that is a consequence of the virus's evolution into four different serotypes. Primary dengue infection causes an unpleasant, but rarely fatal, influenza-like illness that results from the transient release of proinflammatory cytokines from virus-infected monocytes and macrophages. Viremia is already declining by the time of symptom onset, and the illness resolves uneventfully. The recovered individual is thenceforth resistant to reinfection by that serotype. In a small percentage of cases, however, reinfection by a second serotype results in severe disease in which viremia persists and high levels of Interleukin 6 (IL-6), Tumour Necrosis Factor alpha (TNF-α), and other mediators in plasma induce vascular leak and shock (29). Two immune mechanisms are thought responsible for the occurrence of dengue HF: nonneutralizing antibodies resulting from a previous infection are believed to enhance viral replication by linking virions to Fc receptors on the surfaces of target cells, which then take them up into the cytoplasm, thus increasing the number of infected cells, the number of viral particles that enter each cell, and the release of cytokines and other vasoactive mediators. Second, cross-reactive memory CD8+ T cells can attack monocytes and macrophages expressing viral epitopes on their surfaces, triggering an explosive inflammatory response.

Although early discussions of the pathogenesis of VHF attributed vascular leak and hemorrhage to viral infection or injury of the endothelial cells of blood vessels, it now appears more likely that alterations in vascular function and the development of coagulopathy represent physiological responses to circulating proinflammatory mediators. In VHF, the release of large amounts of these substances into the plasma causes vascular dilatation and increased permeability, with catastrophic effects on intravascular volume and blood pressure. This pathogenic process does not mean that viral infection of blood vessel linings cannot also occur. Infected endothelial cells have been observed, for example, in tissues from persons with fatal cases of CCHF (30). Despite the syndrome's dramatic name, hemorrhage is generally a minor feature of VHF. Instead, as in other severe inflammatory syndromes such as septic shock, the major pathophysiological lesion is an increase in vascular permeability ("capillary leak") brought about by mediators released from infected cells. Careful physiological management to maintain sufficient blood flow to critical organs is therefore a hallmark of patient care. The recognized hemorrhagic tendency may be related to decreased synthesis of coagulation and other plasma proteins because of hepatocellular necrosis and the development of a disseminated intracellular coagulation picture. However, massive blood loss is infrequent in EVD, and as in CCHF, confined mainly to the gastrointestinal tract.

CLINICAL MANIFESTATIONS

Most of the signs and symptoms of VHF reflect the release of proinflammatory mediators from virus-infected cells and their effects on temperature regulation, cardiac function, gastrointestinal tract motility, control of vascular tone and endothelial permeability, and the blood clotting system. Fever, malaise, myalgia, and headache are typical early manifestations that tend to evolve insidiously in the case of Lassa fever but can begin so abruptly in YF, CCHF, and the filovirus diseases that patients can report the hour of onset. Fever is usually high and often unremitting; however, it may be absent in some subgroups of patients, including pregnant women and the elderly (31). Bradycardia may be notable, particularly in arenavirus and filovirus diseases and in YF. Vomiting and severe diarrhea frequently occur in filovirus HFs, emphasizing the need for early supportive care often including the administration of intravenous fluids. Abdominal pain can be sufficiently prominent to lead to surgical intervention; hospital-based outbreaks of CCHF and Ebola HF have begun in this fashion. Bleeding rarely is the presenting symptom, but it can occur, e.g., epistaxis in CCHF, which is one reason why the health care workers must be aware of the risk factors and presentation of these diseases to prevent nosocomial spread.

Vasodilatation and increased permeability of the endothelial linings of blood vessels are manifest in a number of physical signs. Capillary dilatation is often signaled by diffuse erythema of the skin of the upper trunk and face that blanches on pressure. Conjunctival injection with petechial hemorrhages is common. An erythematous rash is characteristic in early Marburg, Ebola, CCHF, and dengue, but may be difficult to see in dark-skinned persons. Edema of the face and sometimes of the extremities, observed in Lassa fever, is another manifestation of a capillary leak syndrome, but it may only become evident when a severely ill, dehydrated patient is treated with intravenous fluids. The development of coagulation defects leads to easy bruising, failure of venipuncture sites to clot, hemorrhage from the gastrointestinal and urinary tracts, and menorrhagia. Large ecchymoses are a characteristic sign of CCHF but are rare in the other diseases. Although massive bleeding can occur in severely ill or moribund patients, it is much less common than popular descriptions of these diseases would suggest.

Other clinical manifestations reflect the involvement of specific organ systems. Some degree of hepatic infection and damage is seen in most types of VHF, but the severity varies markedly among the different diseases. Serum levels of aspartate and alanine aminotransferase are markedly elevated in filoviral HF, YF, and RVF, but clinical jaundice is commonly observed only in the latter two conditions. Higher serum aspartate than alanine aminotransferase levels indicate sources other than liver alone. Cough, dyspnea, low oxygen saturations, and the need for mechanical ventilation have all been described in EVD, and difficulty breathing has been associated with a fatal outcome in patients with EVD due to pulmonary edema (32). Gross pathological findings in Lassa fever deceased patients include pulmonary edema.

Renal dysfunction is common in some VHFs. In EVD this may be due to a number of reasons, including hypovolemic shock, acute kidney injury, rhabdomyolysis, and interstitial nephritis. (33, 14) A number of the repatriated cases from the West African EVD outbreak received renal support. The renal compromise phase of HFRS is characterized by severe oliguria with increased blood urea nitrogen and creatinine levels; a diuretic phase typically follows.

The systemic inflammation of VHF leads to numerous changes in clinical laboratory tests, none of which are specific to these diseases. Blood leukocyte patterns vary widely. Leukopenia is frequently observed, but patients with dengue HF usually have normal white blood cell counts. Leukopenia is minimal in Lassa fever, and fatal disease may be heralded by a frank polymorphonuclear leukocytosis. Thrombocytopenia is a universal finding, but it is usually not severe enough in itself to account for hemorrhagic manifestations. Signs of hemoconcentration include a rise in the hemoglobin, hematocrit, and plasma protein levels. Monitored sequentially, these can provide an index of the loss of plasma volume resulting from the capillary leak syndrome and the efficacy of therapeutic countermeasures. Proteinuria is commonly found in VHFs and may be severe in Lassa, Marburg, and Ebola fevers as well as in HFRS. Coagulation factors are variously reduced, but major increases in prothrombin and partial prothrombin times are common only in YF, CCHF, RVF, and the filoviral diseases. Limited testing during outbreaks has shown that fibrin split products and d-dimers, indicative of disseminated intravascular coagulation, are present in EVD and CCHF; they would probably be found in many of the other diseases if testing capability were available.

VHFs can lead to severe complications during pregnancy, leading to fetal loss and life-threatening illness of the mother. In some VHF, including Lassa and filovirus infections, third trimester infection is associated with extremely high rates of maternal and fetal mortality, which may approach 100%. The range and degree of outcomes following infection in pregnancy has not been well explored for all VHFs, including such widespread diseases as dengue. This is an important topic for investigation in future outbreaks of all VHFs.

A range of neurological complications including visual disturbance have been described as a result of RVF infection. These may occur as early or late complications. Examples include encephalitis and loss of vision. The retinal vasculitis of RVF can result in permanent loss of central vision (34, 35, 36, 37).

A few of the VHFs have well recognized sequelae. As in Marburg Virus Disease, EVD has been noted to cause uveitis, with live virus being isolated in some instances from the aqueous. In fact, a range of ocular symptoms was described prior to the West African outbreak, in the Kikwit outbreak in 1995 and the Gulu EVD outbreak in 2001. About 3% of Lassa fever survivors have permanent eighth-nerve damage, making it the most common cause of deafness in young people in its region of endemicity. HFRS has led to sequelae from severe complications occurring during the acute phase of the illness, such as intracranial hemorrhage or renal rupture. Persistence of viral replication in immunologically protected "sanctuary" sites may lead to late sequelae, recrudescence of illness, or late transmission through sexual or other routes, with a risk of rekindling outbreaks, as recently observed for Ebola. The recent Ebola epidemic in West Africa has led to the recognition of a range of sequelae and chronic health impacts, which were not previously well characterized, including arthralgia, new visual problems, and hearing impairment. (38, 39) This recognition has led to continuing research to more carefully characterize the long-term impacts and persistence of infection among large cohorts of survivors. Careful, prospective follow-up of recovered patients has not generally been performed for many other VHFs, therefore generalizations about lack of sequelae should be avoided.

DIAGNOSIS

The signs and symptoms of VHF resemble those of a wide range of infectious diseases, so a specific diagnosis can be made only by means of specialized laboratory tests that directly identify the pathogen. This is particularly true in the early stages of infection, when institution of the correct disease control measures and the appropriate patient management strategy can protect the community and yield the best outcome for the individual patient. Until recently, such assays were generally not performed by hospital laboratories, and required a containment reference facility. Advances in diagnostic technologies have permitted the development of diagnostic platforms and sensitive assays for the suspect VHFs, which can be performed by operators who do not possess advanced laboratory skills, for use in hospitals and field laboratories. Mobile laboratories have been employed which can be easily transported to outbreak sites lacking appropriate laboratory infrastructure and basic services. In the recent EVD outbreak in West Africa, even virus sequencing capacity was brought to the field. However, such capacity is not often on hand at the beginning of an outbreak, and often a combination of clinical and epidemiologic findings will raise the suspicion. For certain VHFs, this may sometimes be in the context of recognition of disease in animals (e.g., waves of abortion in livestock for RVF), or due to an occupational association with animals or their slaughter. Only in rare circumstances in developing countries will the clinician evaluating an acutely ill patient proceed directly to requesting specialized tests, as the symptoms and early clinical features of most VHFs are nonspecific and may be similar to many other endemic illnesses. VHF would be high on the list of likely diagnoses when a patient reports having been exposed to an ill person or a cluster of ill persons, or during an identified VHF outbreak.

A travel and exposure (e.g., animals, ticks, ill persons, healthcare settings) history should always be elicited in returning travelers; more often the patient's recent travel history will only suggest a range of possibilities, rather than a specific diagnosis. Although someone who develops fever and malaise soon after returning from central Africa could be infected with Ebola or Marburg virus, it is much more likely that he or she has a more common (and easily treatable) disease, such as malaria, typhoid fever, or shigellosis. VHF risk has been conservatively estimated at <1 in 1 million

travel episodes to African countries where infection is present, and febrile patients returning from these countries are at least 1,000 times more likely to have malaria than Lassa fever or another VHF (40). Yet, cases of imported VHF have been missed or misdiagnosed in returning travelers (41). The consequences both of missing a treatable infection and missing a diagnosis of a VHF are very serious, so to follow a diagnostic algorithm which considers both is important. An appropriate diagnostic strategy for the clinician faced with a possible case of VHF would therefore be to obtain advice regarding appropriate and safe sample collection to diagnose the most likely diseases, based on the physical examination and history (including the travel history), and assessment of the epidemiological context. Advice should also be sought on how to handle, package, and transport the samples to a laboratory able to perform the necessary tests under the correct biosafety conditions.

A variety of diagnostic tests have been developed for VHF. Viral antigens and antibodies are now detected most commonly by enzyme-linked immunosorbent assay, often employing recombinant antigens and monoclonal antibodies as defined reagents. Molecular tests like reverse transcription polymerase chain reaction (RT-PCR) are being utilized with great success to identify virus-specific sequences. Because many types of VHF are characterized by prolonged viremia, blood is generally considered to be the best sample for testing. However, oral fluid and oral swabs have been used with good results for detecting filoviruses, and oral fluid has been used for diagnosis of Lassa fever. RT-PCR is not the perfect solution for all VHF diagnostics, and indeed for hantavirus infections, in which the viremia occurs early and is short lived in relation to clinical symptoms, serology proves more useful. This may also sometimes be the case for dengue and some other flavivirus infections, in which the viremia may have decreased below the detection limits by the time the patient presents to a health care facility (42).

There have been recent advances in the development of true point-of-care (PoC) tests, performed at the bedside by health workers without specialized laboratory skills. PoC tests with panels to distinguish the potential differential etiologies are in development and should be available for clinical diagnosis and surveillance in the not-too-distant future. Information on diagnostic assays for various types of VHF, including the collection, processing, and disposal of diagnostic specimens, can be found in the appropriate chapters of this book and on a number of infectious-disease websites. In the United States, further information can also be obtained by contacting the Viral Special Pathogens Branch, Centre for Disease Control (CDC), and contact numbers can be found on that webpage (http://www.cdc.gov/ncezid/dhcpp/vspb/diseases.html).

CLINICAL MANAGEMENT

Infection Prevention and Control

The first priority in treating a patient with VHF is to prevent the further spread of infection. The risk of person-to-person transmission varies greatly for the different diseases. Although respiratory spread appears to be rare for most types of VHF, a hospital outbreak of Lassa fever occurred in Nigeria in which a patient in an open ward had a persistent cough. The Andes strain of hantavirus has also been transmitted from patients to medical personnel or family members (43). Several diseases characterized by prolonged, high titer viremia, including Lassa, Ebola, and Marburg fevers and CCHF, can be spread through direct contact with body fluids. In the case of the filoviruses, the body fluids posing a transmission risk includes blood, saliva, vomitus, feces, semen, breast milk, amniotic fluid, and even sweat. The risk of transmission is obviously highest when a specific diagnosis has not yet been made and family members and medical personnel are not taking precautions to avoid contamination.

Whatever type of VHF is being treated, medical personnel must observe standard precautions in handling the patient and diagnostic specimens, and take special care to avoid exposure to aerosolized material, whether during patient care procedures or in the laboratory. Gloves, gowns, and other standard protective measures against blood-borne or enteric diseases should be supplemented with face or eye protection when relevant. The choice of face mask or respirator will be defined by WHO or national agencies and affected by availability. Particular attention should be paid to potential aerosol-generating procedures. The patient's room should be under negative directional air pressure, if possible. Up-to-date advice on the disposal of clinical waste and other aspects of biohazard management during patient care is available from WHO and the U.S. Centers for Disease Control and Prevention.

Supportive Care

Therapeutic interventions for VHF can be divided into general supportive and virus-specific measures. The West African Ebola outbreak experience, including use of intensive monitoring and supportive care (e.g., blood products, renal replacement therapy, and mechanical ventilation) in some treatment centers, and also in small numbers of repatriated health care workers, has resulted in a deeper understanding of the supportive medical care required by EVD patients (44, 11). General supportive management should, whenever possible, be based on careful monitoring of circulating blood volume and correction of electrolyte abnormalities (45, 46, 47). Inotropic agents and vasopressors may also be indicated. During VHF outbreaks where malaria coinfection is common, MSF's recent experience with artesunate-amodiaquine would suggest that artesunate–amodiaquine is preferable to artemether–lumefantrine in patients with confirmed EVD (48). Amodiaquine has some *in vitro* activity and there are concerns over QT intervals and lumefantrine in patients who are likely to be hypokalemic and hypomagnesemic due to diarrhea and vomiting. Although corticosteroids in various doses have been administered to patients with many types of VHF, there is no evidence that they are of benefit for any of these conditions, and should be avoided unless required for other medical indications like adrenal support. It is also advised the Non Steroidal Anti Inflammatory Drugs (NSAIDs) and aspirin are avoided for risk of bleeding, and some patients have impaired renal function.

Antiviral Treatment

Specific therapeutic measures are available for only a few of these diseases and there remains doubt over the efficacy of these drugs. Intravenous administration of the guanosine analogue ribavirin appeared to improve the survival rate in severe Lassa fever in a clinical trial in Sierra Leone in the 1980s (49), and treatment has also reduced the rate of mortality from HFRS (50). However, two systematic reviews have concluded that insufficient evidence exists to support the claim of therapeutic efficacy of ribavirin in CCHF (51, 52). Ribavirin has been used for postexposure prophylaxis of CCHF in health care workers (53).

Convalescent plasma with uncertain specific antibody content did not appear effective in reducing mortality in EVD (54). However, convalescent plasma with neutralizing

antibodies has been successfully used in Argentine HF. A placebo-controlled trial in the late 1970s showed that such therapy significantly reduced the case fatality rate, providing it was initiated before the eighth day of illness (55). Yet in contrast to untreated patients who undergo a typical HF syndrome, patients who receive immune plasma sometimes relapse a few weeks after the completion of therapy with a variety of neurologic abnormalities. The etiology of this "late neurologic syndrome" is unknown and merits further study as these therapies are often some of the first to be considered (55). Despite promising results from the use of convalescent plasma in Lassa in Nigeria in 1984 (56) subsequent laboratory studies explored the titer levels and the need for a good match between the patient and plasma donor. Once ribavirin was thought to be beneficial in Lassa, the option of convalescent plasma was largely abandoned.

A number of experimental therapies have shown efficacy in laboratory animal models of various types of VHF, as described in the corresponding chapters of this book. Most of the drug development efforts over the years have gone into Ebola and a number of these therapeutics were trialed in the recent West African outbreak (see chapter 42). However, no individual drug (e.g., favipiravir) or immunotherapeutic (e.g., ZMapp) has demonstrated convincing antiviral or therapeutic benefits in rigorously controlled trials (58), in part because studies were under-enrolled and/or observational in nature. Several of these compounds were also used in expatriated patients, but for multiple reasons, including administration of multiple therapies, no conclusions can be drawn about their efficacy (14). One recently described prodrug of an adenosine analogue, designated GS-5734, is a potent inhibitor of Ebolavirus replication *in vitro* and in experimentally infected Rhesus macaques (59); this agent was used in several cases with apparent benefit.

If these approaches prove to be safe and effective in humans, they could have many applications in the setting of a disease outbreak, and could be lifesaving for a laboratory worker accidentally exposed to a pathogen. In contrast, developing an effective treatment for full-blown VHF, with its combination of vascular insufficiency, disseminated intravascular coagulation, tissue damage, and impaired immune function, is likely to remain a challenging medical problem for the foreseeable future.

PREVENTION

It is impossible to prevent all sporadic cases of VHFs, especially those involving spillover from wildlife or a domesticated animal source. Early detection and response remain fundamental to preventing the extension of outbreaks from such events. This entails the strengthening of surveillance systems, health care worker awareness, diagnostic capacity, and adherence to the International Health Regulations (2005). Local health care workers should be trained to recognize the signs and risk factors of early VHF infection. Analyses of the weaknesses in controlling the West Africa Ebola epidemic noted the need for improved national surveillance and response systems, as required by the International Health Regulations (IHR), along with strong global leadership and coordination in responding to such events.

Transmission Reduction Strategies

For VHFs like EVD that can have delayed sexual transmission, it is important that WHO recommendations are followed regarding safe sex practices, including sexual abstinence or condom use until semen has tested negative on two occasions with an identified interval. Ideally, men should be offered testing of their semen regularly for Ebola RNA from 3 months after the onset of symptoms. If not possible, then precautions should continue for at least 6 months after the onset of symptoms. For vector-borne VHFs personal protection should be used when possible, including mosquito repellants. For Crimean Congo Haemorrhagic Fever Virus (CCHFV) and ticks, animal husbandry practices should be reviewed as animals often roam easily amongst tick-infested grassland and towns. Safe and quick removal of ticks should be taught to all at-risk communities. During VHF outbreaks, transmission-based precautions should be taken to avoid contamination of the blood supply.

Vaccines

Only a few vaccines are in regular use in humans for the prevention of VHF. The live attenuated 17D YF vaccine, first introduced in the 1930s, remains one of the most effective vaccines ever developed, but its use in tropical countries, where sylvatic infection is endemic, is unfortunately insufficient to prevent recurrent epidemics as is the current situation in sub-Saharan Africa with outbreaks affecting a number of countries. Effectiveness of reduced dosing of the vaccine is currently being studied in the attempt to make it available to more people. The attenuated Candid-1 vaccine for Argentine HF has also proven to be highly effective and has played an important role in reducing the incidence of disease in its zone of endemicity. Some older vaccines against RVF are used for specified groups of at-risk humans in very limited settings in a few countries. A vaccine against Omsk HF is used for high occupational risk persons in the endemic area. The most problematic disease from the point of view of vaccine development has been dengue fever. As discussed above, the immunity that follows infection by one of the four dengue virus serotypes can predispose an individual to severe disease if he or she is later exposed to a second serotype. So as not to place vaccinees at risk of severe illness, it is now generally accepted that a dengue vaccine must protect against all four serotypes (60). One dengue vaccine has recently completed clinical evaluation; the current status is described in chapter 53.

Several vaccines were fast tracked for evaluation against Ebola during the West African epidemic. These included recombinant vesicular stomatitis virus (rVSV) and chimpanzee adenovirus vectored vaccines expressing the Ebola surface glycoprotein. Both of these have been demonstrated to be safe and immunogenic, but due to declining case numbers, only the rVSV vaccine has gone far enough to yield interim results indicating protection against EVD (61). These vaccines, and other candidates, must be evaluated for efficacy in future outbreaks, but safety and immunogenicity studies can be carried out in the intervals between outbreaks, so that the necessary clinical trials can be initiated with minimal delay when the outbreaks begin. Some of the newer vaccine technologies, which were explored for Ebola, may also be adaptable to other VHFs, and work is under way to assess these vaccine platforms for application to one or more of the other VHFs mentioned in this chapter.

The various disease control needs, such as vaccination for VHF outbreak control or the vaccination of potential frontline workers or other at-risk groups, may necessitate different vaccination strategies, and indeed, different vaccines. Immediate use to curtail an outbreak demands a one-dose schedule with rapid induction of immunity. Protection of frontline workers or populations facing repeated exposure over a long time period may require multiple doses or combination of vaccines, e.g., a prime-boost strategy. In addition, while there

are a variety of candidate vaccines for VHFs, the absence of a lucrative commercial market to drive their development and manufacture has led to slow progress in moving them through the development pipeline. One consequence of the West Africa Ebola epidemic was increased awareness of a need for better available vaccines and vaccine preparedness at the global level. It is hoped that this will translate into the attention and funding required to accelerate their progress and to make them available when and where they are needed.

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Viral Disease of the Eye

HOWARD M. NEWMAN

10

The eye is a fascinating organ not only because of its complex anatomy but also because it is a partly immunologically privileged site, which protects itself from potentially destructive systemic immune responses. This characteristic, however, may hinder defense against infectious agents, including numerous viruses, which may manifest with a variety of ocular diseases (Table 1). The first part of this chapter discusses ocular anatomy and physiology, as well as the principle clinical syndromes, which are usually classified according to the affected anatomic structures. The latter part discusses the major viral ocular pathogens and highlights selected risk groups, such as infants and patients infected with human immunodeficiency virus (HIV).

ANATOMY AND PHYSIOLOGY

Figure 1 illustrates the anatomy of the eye, which is a complex organ within which three layers can be distinguished. The outer layer consists of the cornea and sclera. The middle one consists of the iris, ciliary body and choroid, collectively known as the uvea. The retina forms the inner layer of the eye and is a complex multilayered sheet of neural tissue closely applied to a single layer of pigmented epithelial cells (1, 2). The three transparent structures surrounding these layers are the aqueous, the vitreous, and the lens. The eye can also be divided into anterior and posterior segments, with the dividing line just behind the lens (3).

The cornea, which is the window of the eye, is not only transparent but also avascular; it consists of five distinct layers: the corneal epithelium, Bowman's membrane, stroma, Descemet's membrane, and the corneal endothelium. The surface of the cornea is covered by tear film, which protects against foreign substances, including infectious agents (1, 2). There are several antimicrobial substances in tear film, such as lactoferrins and lipocalin, which may interfere with viral attachment to cells, as well as immunoglobulins (IgG and IgA), which may neutralize viruses, thus preventing ocular infection (4). All these characteristics allow the cornea to perform its main function: to refract and transmit light to the lens and retina and to protect the eye against infection and structural damage to deeper parts (1).

The sclera forms a connective tissue coat which helps maintain the shape of the eye. It contains collagen fibrils arranged haphazardly, and therefore it is opaque rather than clear. The outer layer of the sclera is the episclera, a highly vascular connective tissue (2). The cornea and the sclera connect at the limbus, while the conjunctiva covers the visible part of the sclera. The iris (in the middle layer) controls the size of the pupil, thereby controlling the amount of light entering the eye. The ciliary body controls the power and shape of the lens and is the site of aqueous production, while the vascular choroid provides nutrients to the outer retinal layers (1). Inflammation of the uvea (uveitis) would therefore affect these functions to varying degrees. Aqueous humor contains components of both innate and adaptive immunity but also contains several substances, such as the cytokine TGF-beta 2 (transforming growth factor), which may suppress the activation of T-helper cells, thus contributing to immune privilege (5).

The retina, the tissue that lines the inner surface of the eye, is an essential ocular component with its primary purpose being photoreception. It surrounds the vitreous cavity containing the vitreous humor. Like the aqueous humor, the vitreous humor is also subject to innate and adaptive immune responses, which protect against infection, and immune privilege is also present in the vitreous (6).

Adnexal structures such as the eyelid, periocular skin, and lacrimal glands and associated structures are often overlooked when considering the anatomy of the eye, yet they are important sites for many viral diseases.

In the eyelid and conjunctival sac, immune cells line the sites at which ocular exposure to antigens occurs. Lymphocytes and antigen-presenting cells of the conjunctival-associated lymphoid tissue (CALT) form a distinct layer in the substantia propria and, in places, cluster to form follicles. The CALT is considered part of the mucosa-associated lymphoid tissue. These tissues are protected primarily by immunoglobulin A (IgA) antibodies and T-cell-mediated immune responses (7).

Visual function is critically dependent on normal structural integrity, as functional repair processes are incapable of remodeling all ocular components if extensive tissue damage occurs, which sometimes may be a direct result of an overzealous host immune response. To reduce this likelihood, the eye has evolved mechanisms, which evoke specific immunological adaptations. Theses adaptations have been termed anterior chamber-associated immune deviation, or "immune privilege," and consist of suppression of relatively

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TABLE 1 Common viruses affecting the eye

Virus	Family	Transmission
HSV-1	*Herpesviridae*	Close personal contact
VZV		Respiratory droplets
CMV		MTC, kissing, sex, blood transfusions, transplant recipients
EBV		Contact with infected saliva
HIV	*Retroviridae*	Sexual intercourse, MTC, needle-stick injuries
Adenovirus	*Adenoviridae*	Faecal-oral, aerosol inhalation → depends on type of adenovirus
Enterovirus	*Picornaviridae*	Faecal oral, respiratory route, direct contact
Measles	*Paramyxoviridae*	Respiratory aerosols
Rubella	*Togaviridae*	Respiratory droplets

HSV, herpes simplex virus; VZV, varicella zoster virus; CMV, cytomegalovirus; EBV, Epstein Barr virus; HIV, human immunodeficiency virus; MTC, mother-to-child.

nonspecific, delayed-type hypersensitivity responses, with preservation of specific noncomplement fixing humoral immunity and up-regulation of specific T-cell responses. In addition, blood-ocular barriers (tight cellular junctions of capillary endothelial cells and retinal pigment epithelium) in the retina and anterior segment, limit ocular immune responses by restriction of cellular macromolecular traffic, and programmed intra-ocular apoptosis of inflammatory cells may occur. These immune modulations and adaptations modify the severity and extent of nonspecific intraocular inflammation and serve to reduce collateral damage (7).

MAJOR CLINICAL SYNDROMES

Table 2 illustrates the major clinical syndromes, as well as their viral etiologies and special features.

Conjunctivitis

Conjunctivitis refers to inflammation of the conjunctiva and has four main causes—viruses, bacteria, allergens, and irritants (8)—with viral causes being the most common. Viral causes of conjunctivitis are numerous, with adenovirus being one of the more common etiologies. In addition, conjunctivitis often occurs in association with keratitis (keratoconjunctivitis).

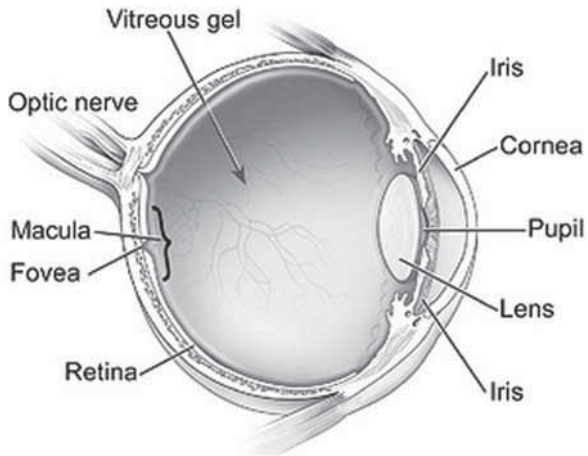

FIGURE 1 Anatomy of the eye. Courtesy of the National Eye Institute, National Institutes of Health, USA.

Conjunctival inflammatory responses are divided into nonspecific papillary responses (as a consequence of tissue edema) and follicular responses (due to the formation of aggregates of activated lymphocytes) (7). In severe cases, transudates rich in protein and fibrin may coagulate to form membranes and pseudomembranes on conjunctival surfaces.

Clinical features include mild pruritus, foreign body sensation, matted eyelids, generalized conjunctival injection, and discharge (8,9).

Keratitis

Keratitis refers to inflammation of the cornea. Herpes simplex virus (HSV), in particular, may cause severe keratitis resulting in blindness (10). However, numerous other viruses, such as adenovirus and measles, may also present with corneal manifestations. The resultant clinical syndrome will depend on the anatomical location of the lesion. Epithelial disease may present with dendritic or amoebic/geographical ulcers, whereas stromal disease may present with disciform or interstitial keratitis. Significant inflammation affecting the cornea results in limbal vascular dilatation with erythema, most marked at the corneo-scleral limbus (7).

Symptoms include ocular pain, which is usually moderate to severe, photophobia, which is thought to be due to reflex inflammation and spasm of the iris in response to light, and a watery discharge as a result of reflex lacrimation and mucous buildup. Visual compromise may occur as a consequence of reactive blepharospasm or, more significantly, inflammatory cell infiltration and structural damage and perforation (7).

Early and accurate diagnosis with effective treatment is essential if permanent damage is to be avoided. To this end, specific laboratory diagnosis is useful.

Scleritis and Episcleritis

Scleritis is a severe ocular inflammation that is often associated with more severe ocular complications and is nearly always treated with systemic medications. In patients with scleritis, the vasculature is engorged, whereas in the healthy eye, the scleral vessels are not prominent (11, 12). If the anterior sclera is involved, the external surface of the eye becomes red and tender, with the redness being diffuse or localized. Anterior scleritis can therefore be divided into diffuse, nodular or necrotizing types, with the latter being less common. Posterior scleritis is rare and may present with pain and decreased vision.

Episcleritis is inflammation of the tissue that lies immediately superficial to the sclera, deep into the conjunctiva. Differentiation of episcleritis and scleritis, which may be

TABLE 2 Viral causes of ocular clinical syndromes

Clinical syndrome	Virus	Features
Conjunctivitis	Adenovirus	Follicular, pseudomembranous/membranous
	HSV	Follicular conjunctivitis, neonatal ophthalmia
	VZV	Mucopurulent conjunctivitis with lid margin lesions
	EBV	Follicular or membranous conjunctivitis
	Measles	Keratoconjunctivits with conjunctival Koplik's spots
	Mumps	Follicular or papillary conjunctivitis
	Enterovirus	Acute haemorrhagic conjunctivitis
	Influenza	Papillary conjunctivitis
	Molluscum contagiosum	Follicular conjunctivitis as reaction to shedding of molluscum bodies into tear film
Keratitis	HSV	Epithelial or stromal
	VZV	Primary infection → punctuate epithelial keratitis, disciform keratitis
		Reactivation → epithelial or stromal keratitis
	Adenovirus	Subepithelial punctate opacities
	Measles	Epithelial keratitis → usually in malnourished or immunocompromised children
	Mumps	Punctate epithelial keratitis, disciform keratitis
	Rubella	Punctate epithelial keratitis, stromal keratitis
	Vaccinia	Epithelial, interstitial, or stromal keratitis
Scleritis / Episcleritis	HSV	Scleritis or episcleritis
	VZV	Scleritis or episcleritis
	Mumps	Scleritis or episcleritis
	Influenza	Episcleritis
	EBV	Scleritis or episcleritis
Uveitis	HSV	Anterior uveitis and possibly sectorial atrophy
	EBV	Anterior uveitis
	VZV	Anterior uveitis
	Adenovirus	Rarely, anterior uveitis
	Mumps	Anterior uveitis
	Influenza	Anterior uveitis
	Vaccinia	Anterior uveitis, choroiditis
Retinitis	CMV	Necrotizing retinitis, usually in AIDS patients
	HIV	Retinopathy/ Microvasculopathy
	HSV`	Acute retinal necrosis
	VZV	Necrotising herpetic retinitis
Ocular adnexal disease	HSV	Blepharitis with primary infection
	VZV	Lid cicatrisation with HZO
	Molluscum contagiosum	Molluscum eyelid nodules
	Papillomavirus	Papillomas on lid, conjunctiva
	HHV-8	Kaposi Sarcoma

HSV, herpes simplex virus; VZV, varicella zoster virus; EBV, Epstein Barr virus; CMV, cytomegalovirus; HIV, human immunodeficiency virus; HZO, herpes zoster ophthalmicus.

caused by several viruses, can be difficult (Table 2), but correct identification is crucial as the prognosis of episcleritis is better than for scleritis (9). Other than viral causes for these syndromes, there are numerous other systemic and autoimmune conditions which may present with scleritis or episcleritis. These range from rheumatoid arthritis and systemic lupus erythematosis to metabolic conditions, such as gout, plus many others. (13)

Initial treatment of scleritis usually involves systemic anti-inflammatory agents, but cytotoxic agents are sometimes required to control inflammation (7).

Uveitis

Uveitis is the term used to describe many forms of intraocular inflammation involving the uveal tract (iris, ciliary body, and choroid) and adjacent ocular structures (retina, vitreous, and optic nerve) (14). The classification of uveitis into anterior, posterior, or diffuse is based on the physical appearance of the inflamed eye (14).

In anterior uveitis there is often vascular congestion of the conjunctiva and sclera. The infiltration of inflammatory cells between the anterior iris and cornea may result in closure of the anterior chamber angle, resulting in increased

intraocular pressure. Alternatively, intraocular pressure may decrease as a result of uveitis, and aqueous humor production is diminished. Chronic or recurrent anterior uveitis often leads to opacification of the lens.

In posterior uveitis, vision often decreases as a result of opacity formation in the vitreous or from inflammation or vascular occlusions of the macular area of the optic nerve (14).

Anterior uveitis is typically associated with limbal hyperemia, pain, photophobia, and reflex lacrimation (7). Although several viruses are associated with anterior uveitis (Table 2), it is usually associated with rheumatoid-negative spondyloarthropathies, such as ankylosing spondylitis and Reiters syndrome.

Treatment is usually aimed at reducing the ocular inflammation with topical or systemic steroids or nonsteroidal anti-inflammatory drugs, while treating any underlying etiology where applicable.

Retinitis

Infections of the retina are potentially sight-threatening. Viral causes of retinitis have become more prominent with the global AIDS pandemic (7). This is particularly true for cytomegalovirus (CMV) retinitis, which is an AIDS defining illness and usually occurs in patients with severe immunosuppression (15), particularly with compromised cellular immunity. On the other hand, viruses, such as varicella zoster virus (VZV) and HSV, may cause retinitis in immunocompetent individuals (15). Nonviral etiologies include *Toxoplasma gondii*, syphilis, fungal infections, and tuberculosis, whereas noninfectious causes include autoimmune conditions, such as sarcoidosis, as well as inherited conditions, such as retinitis pigmentosa, all of which need to be considered in the differential diagnosis of retinitis.

Retinal inflammatory cellular infiltrates and retinal ischemia may result in damage to blood-retinal barriers, which may allow inflammatory cells to enter the vitreous cavity and produce opacities, giving rise to symptoms of visual floaters (7).

Acute retinal necrosis (ARN), classically described in immunocompetent individuals, may involve one or both eyes and may be characterized by anterior uveitis, vitritis, and retinal vasculitis with diffuse or patchy areas of retinal necrosis (15). In contrast, progressive outer retinal necrosis (PORN) is seen mainly in immunocompromised patients and is characterized by outer retinal discoloration in the posterior pole, which rapidly spreads throughout the fundus (15, 16). Together, ARN and PORN are classified as necrotizing herpetic retinopathies (NHR) (3), with the primary etiologic agents being VZV and HSV 1 and 2, although CMV and Epstein–Barr virus (EBV) are also implicated (7, 15, 17). The visual prognosis for NHR is poor, especially when caused by VZV (18).

Ocular Adnexal Disease

The ocular adnexa comprise anatomically related tissues such as the eyelids, lacrimal apparatus, the extraocular muscles, and periocular skin. Numerous viruses result in adnexal disease. Disease features depend on the structures involved and are beyond a general description.

VIRAL OCULAR PATHOGENS

HIV and the Eye

By 2015, over 35 million people worldwide were living with HIV (see Chapter 34). As access to lifesaving antiretrovirals increases, this number is expected to steadily rise (19). The impact that this pandemic has had on all specialties within medicine, ophthalmology included, has been immeasurable. Eye clinics in developing countries, in particular, where the prevalence of HIV is high, have been put under immense strain to provide clinical care. This is not surprising, since HIV can affect the eye directly, or, by causing severe immunosuppression, make the eye susceptible to a variety of devastating infectious diseases, including other viral infections. As such, HIV has to be considered a major ocular viral pathogen.

TABLE 3 Ocular HIV manifestations

Category	Features
Microvasculopathy	HIV retinopathy
	Conjunctival microvasculopathy
Opportunistic infections	Viral → CMV, HSV, VZV, Molluscum
	Bacterial: MTB, Syphilis
	Fungal: Candida, Aspergillus
	Protozoan: *Toxoplasma gondii*, Microsporidia
Neoplasms	Non-Hodgkin lymphoma
	Kaposi sarcoma
Optic neuropathies	Optic neuritis (eg HHV-6)
	Compression of optic nerve due to neoplasm

HIV, human immunodeficiency virus; CMV, cytomegalovirus; HSV, herpes simplex virus; VZV, varicella zoster virus; MTB, *Mycobacterium tuberculosis*; HHV-6, human herpes virus-6.

Eye infections tend to be more severe and are more commonly multicentric and bilateral. Approximately 70% of AIDS patients will develop eye disease during the course of their illness (20). HIV ophthalmic manifestations, illustrated in Table 3, fall within four major groups: microvasculopathy, opportunistic infections, neoplasms, and neuro-ophthalmic disorders (21). Anterior segment findings include keratitis, keratoconjunctivitis sicca, iridocyclitis, and others. Posterior segment findings include HIV-associated retinopathy and opportunistic infections of the retina and choroid (20).

Microvasculopathy, which is the most common form of posterior segment involvement in AIDS patients, may affect the conjunctiva or retina, with the latter also referred to as HIV retinopathy (22, 23). It is hypothesized that the pathogenesis of conjunctival and retinal microvasculopathy is possibly similar and may include increased plasma viscosity, circulating immune complexes and infectious damage of the vasculature (3, 20).

Conjunctival microvasculopathy, which may result in asymptomatic microvascular changes, usually requires no treatment (20). The microvascular changes include microaneurysms and vascular dilatations and narrowings (7, 20).

Although being the most common retinal manifestation of HIV, most cases of HIV retinopathy are asymptomatic. Fundoscopic changes (Figure 2) include cotton wool spots, intraretinal hemorrhages and retinal micro-aneurysms (3, 24). The possible opportunistic infections that may affect the eye are numerous (Table 3) and include viruses, bacteria, fungi, and protozoa (25). Among the viral opportunistic infections, CMV, HSV, and VZV can cause severe disease, especially in those with severe immunosuppression, and are discussed in more detail in the sections to follow.

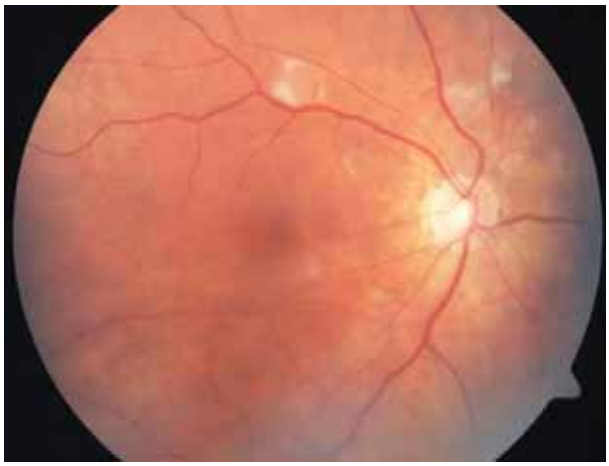

FIGURE 2 HIV retinopathy with cotton wool spots. Courtesy Retinal Gallery and S Cohen (http://retinagallery.com/displayimage.php?pid=3301).

Orbital and adnexal manifestations of HIV are rare, with the most common being opportunistic viral infections, such as molluscum contagiosum, as well as HIV-associated conditions such as Kaposi sarcoma and conjunctival microvasculopathy (26). In addition to infectious complications, HIV may cause optic neuropathies by various mechanisms, including compression on the optic nerve by tumors and vaso-occlusion (3). In children, ocular manifestations of HIV are much less frequent than in adults for reasons which are still unclear. However, the commonest ocular manifestation of HIV in children is keratoconjunctivitis sicca, or dry eyes (20, 27).

With close to 10 million people worldwide receiving highly active antiretroviral therapy (HAART) by 2012 (28), immune-recovery uveitis is a syndrome expected to occur more frequently. It entails paradoxical worsening of intraocular inflammation after receiving antiretrovirals, which may be caused by an immune response to CMV and other infectious agents present in the eye (3, 27), and is characterized by recovery of CD4 cell counts and function. Although specific treatment of opportunistic infections is also important, the mainstay of treatment for ocular complications of HIV would be HAART, which is discussed elsewhere and is mentioned in the following sections.

Herpesviruses

Viruses of the *Herpesviridae* family have biological features of latency and cytopathic effects, which are important characteristics with respect to ocular manifestations. These viruses may evade the host immune system by a variety of mechanisms, including latency, which allows for persistent, life-long infections, and the potential for reactivation with or without disease (3).

Table 4 shows the various herpesviruses and their common ocular manifestations. Herpes simplex virus 1 and 2 (see chapter 20) (HSV-1, HSV-2), CMV (see chapter 23), and VZV (see chapter 22) are the herpesviruses with the greatest propensity for severe disease, which may or may not result in blindness.

Herpes Simplex Virus

HSV-1 generally enters the host via the oropharyngeal mucosa, where initial replication occurs. This is followed by the establishment of latency in the trigeminal ganglia or sacral ganglia, depending on the mode of transmission. HSV-2, on the other hand, is usually transmitted sexually with initial replication in the genital mucosa, followed by latency in the sacral ganglia. HSV-2 infection acquired congenitally may or may not have ocular manifestations (29, 30). HSV-1 and -2 have similar clinical ocular manifestations and produce a wide spectrum of diseases. However, HSV-1 predominates with regard to ocular infections.

Ocular manifestations of HSV-1 include blepharitis, conjunctivitis, keratitis, uveitis, and retinitis (31, 32, 33, 34), with keratitis being the most common. HSV keratitis is also the commonest cause of infectious blindness in developed countries (35). Keratitis may manifest as corneal edema, epithelial ulceration, or stromal inflammatory infiltrates. Due to the inability of the repair process to precisely remodel the orthogonal collagen fibers of the original corneal structure, inflammatory processes may produce permanent scarring and decreased visual acuity (7).

Pathogenesis

There are two major routes for primary ocular infection: direct infection by contact with infectious secretions or initial infection at a nonocular site with neural spread along the nerves supplying the cornea (36). Autoinoculation appears to be an unlikely source of intraocular infection (37).

Primary infection may be followed by intermittent reactivation (38), both of which may produce ocular disease. Reactivation episodes may be responses to various stimuli including fever, stress, exposure to ultraviolet light, hormonal imbalances, among others, and is made possible by

TABLE 4 Ocular manifestations of herpesviruses

Virus	Extraocular manifestations	Common ocular manifestations
HSV-1	Oral and peri-oral skin lesions, genital lesions, encephalitis	Blepharitis, conjunctivitis, keratitis, uveitis, retinitis
HSV-2	Genital lesions, oral skin lesions, encephalitis	Keratitis, uveitis, retinitis, congenital cataract
VZV	Chickenpox, meningitis, encephalitis, pneumonia, herpes zoster	HZO, keratitis, uveitis, NHR
CMV	Meningitis, encephalitis, pneumonia, hepatitis, colitis, plus others	Retinitis, uveitis
EBV	Infectious mononucleosis, Burkitt's lymphoma, central nervous system (CNS) manifestations	Conjunctivitis, keratitis, uveitis, retinitis
HHV-6	Sixth disease, CNS manifestations, hepatitis	Uveitis, optic neuritis
HHV-8	Kaposi sarcoma	Conjunctival Kaposi sarcoma

HSV, herpes simplex virus; VZV, varicella zoster virus; HZO, herpes zoster ophthalmicus; NHR, necrotising herpetic retinitis; CMV, cytomegalovirus; EBV, Epstein Barr virus; HHV-6, human herpes virus-6; HHV-8, human herpes virus-8.

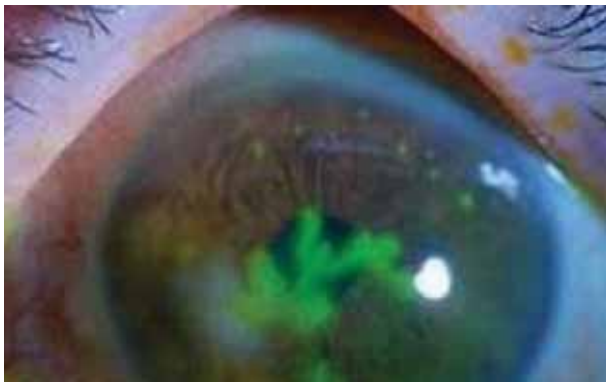

FIGURE 3 Herpes simplex virus keratitis with dendritic ulcer, visible after fluorescein staining.

the virus's ability to remain latent in the sensory ganglia of the first division of the trigeminal nerve.

Infectious epithelial keratitis with dendritic or geographical ulcers is associated with the formation of multinucleated giant cells and intranuclear inclusion bodies, as well as necrosis of the cells bordering the area of ulceration and infiltration with neutrophils in the underlying stroma (7). In stromal keratitis, in addition to the influx of neutrophils, lymphocytes are present and are important in predisposing to chronic inflammatory sequelae. How HSV enters the stroma still needs to be fully elucidated, but it seems likely that virus present in the epithelial cells migrates into the superficial stroma from the subepithelial nerve plexus and then enters the keratocytes, with subsequent dissemination into the stroma through intercellular spread. This spread would be enhanced when immune responses are reduced by corticosteroid use (7, 39).

HSV antigens can be detected in keratocytes, endothelial cells, and epithelioid hystiocytes, together with multinucleated giant cells in necrotizing stromal keratitis. With inactive disease, no viral antigens are apparent. Endotheliitis, on the other hand, is associated with HSV DNA in the aqueous humor. Endothelial cells are dysfunctional due to direct infection with HSV and as a consequence of anterior chamber inflammation.

Clinical manifestations

HSV may cause epithelial ulcerative keratitis, which is generally self-limiting and responds rapidly to antiviral therapy. The typical dendritic ulcer caused by HSV-1 is a form of infectious epithelial keratitis and occurs as a result of active viral replication (Figure 3). Stromal keratitis results in temporary or permanent loss of corneal transparency, and loss of corneal sensation is a useful clinical sign of its occurrence (7).

Primary ocular infection is rare and may manifest as vesicular lesions on the eyelid, follicular inflammatory response in the conjunctiva, or dendritic ulcers in the cornea.

In recurrent disease, stromal involvement may occur, while other manifestations include iridocyclitis, panuveitis, and ARN, the latter being rare and usually occurring in immunocompetent hosts (40).

Epithelial disease causes moderate to severe pain, watering, and photophobia. Infections are usually unilateral, and recurrences are associated with triggers, such as fever, stress, and exposure to sunlight (41).

Dendritic ulcers are narrow and branching and are best visualized after instilling fluorescein drops. Untreated, these ulcers usually heal in 5 to 12 days (7). In malnourished children with measles virus infection, or in cases where corticosteroids were erroneously used to treat a dendritic ulcer, these lesions may evolve into more extensive amoeboid or geographical ulcers (Figure 4).

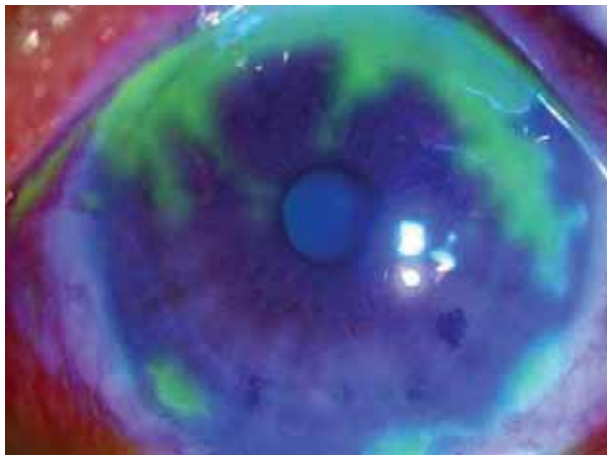

FIGURE 4 Herpes simplex virus keratitis with geographical ulcer. Courtesy of Hee K. Yang, Young K. Han, Won R. Wee, Jin H. Lee, and Ji W. Kwon; Department of Ophthalmology, Seoul National University College of Medicine, Seoul, Korea (http://www.jmedicalcasereports.com/content/5/1/328/figure/F1).

Stromal keratitis is an inflammatory reaction and is the most common sight-threatening manifestation of ocular HSV (42). Spread of viral particles from the epithelium is prevented by relative barriers, such as the basement membrane of the epithelium and Bowman's layer. However, in approximately 30% of patients with ulcerative disease, viruses may still penetrate into the corneal stroma (7, 43, 44). Permanent stromal scarring generally follows. Scarring may not be sufficient to cause permanent visual handicap, but when it affects the visual axis, it may result in significant visual compromise for which a corneal transplant may be required (45).

Diagnosis

HSV keratitis is usually diagnosed clinically, but, in doubtful cases, specific diagnostic tests are indicated. Table 5 illustrates the common laboratory tests for the common ocular viral infections. Detection of viral DNA by polymerase chain reaction (PCR) is more sensitive than viral culture or antigen detection (3, 34). Intraocular infection can be demonstrated by PCR amplification of viral DNA found in the anterior chamber or vitreous fluids. In addition, locally produced antibody may be detected, and, by comparing with total immunoglobulin G (IgG), one can calculate the Goldmann-Witmer coefficient (GWC), which is the quotient of two ratios, namely the specific antibody titer in the aqueous humor over the total IgG in the aqueous humor and the specific antibody titer in the serum over the total IgG in the serum. A value of 3 or more indicates infection with the specific virus involved. Using a combination of PCR and GWC allows for a more comprehensive laboratory diagnosis (46).

Treatment

The goal of treatment is to inhibit viral replication and to reduce the inflammatory reaction in the stroma that may lead to long-lasting damage to the collagen fibrils (47, 48). Topical steroids are never used in isolation for epithelial keratitis because the virus can replicate freely in the presence

TABLE 5 Laboratory diagnosis of common ocular viral infections

Virus	Specimen	Laboratory test options
HSV	Aqueous humour, biopsy specimens	PCR, Goldman-Witmer coefficient (GWC), culture
VZV	Aqueous humour, biopsy specimens	PCR, GWC, culture
CMV	Vitreous humour, biopsy specimens, blood	PCR, culture, serology and viral load on blood (complementary tests)
EBV	Biopsy specimens, blood	PCR, serology on blood (complementary test)
Adenovirus	Aqueous humour, biopsy specimens	PCR, culture
Enterovirus	Aqueous humour, biopsy specimens	PCR, culture
Rubella	Aqueous humour, biopsy specimens, blood	PCR, GWC, serology on blood (complementary test)

HSV, herpes simplex virus; VZV, varicella zoster virus; CMV, cytomegalovirus; PCR, polymerase chain reaction; EBV, Epstein Barr virus.

of local steroid-induced immunosuppression, potentially resulting in more extensive geographical ulcers. Prior to antivirals being available, alternative treatment modalities were used, including cautery with carbolic acid, ether, or iodine (7), which were associated with stromal scarring. Debridement using a cotton-tipped applicator to gently remove loose epithelium is an alternative when antiviral treatment is unavailable (49).

Antivirals

Antiviral agents are the mainstay of treatment in HSV infections. The decision whether to use topical or systemic agents depends on the anatomical site involved, severity of infection, and immune status of the patient (3). Available topical agents include acyclovir and trifluridine, with the former being the most widely used. Systemic agents other than acyclovir includes valacyclovir (the prodrug of acyclovir) and famciclovir.

Topical antivirals are used to treat HSV blepharitis, conjunctivitis, and infectious epithelial keratitis and are also used as prophylaxis to cover corticosteroid treatment of certain forms of keratitis. Systemic agents are used in certain cases of endotheliitis and severe uveitis in immunocompromised patients, in pediatric patients not responding to topical treatment, or as prophylaxis (3). Intravenous acyclovir is generally reserved for patients with posterior pole involvement (30, 33).

For recurrent epithelial or stromal keratitis, as well as corneal graft failure in patients with a history of recurrent HSV keratitis, long-term prophylaxis with oral acyclovir appears to reduce their occurrence (50–52).

Resistance to acyclovir has been reported, particularly in AIDS patients, where isolates were shown to have mutant thymidine kinase (53).

Corticosteroids

The use of corticosteroids to reduce inflammation in HSV ocular disease is controversial, since it may lead to increased severity of HSV infection by suppressing the local immune response. However, in certain cases they are very beneficial in reducing scarring and neovascularization. Topical steroids are usually reserved for moderate to severe cases of stromal keratitis, endotheliitis, and uveitis. Systemic steroids are only used in very severe forms of stromal and endothelial keratitis, uveitis, and retinitis after initiating antiviral therapy (33, 54). High-dose corticosteroids should be tapered over days to weeks to avoid the risk of a severe rebound inflammatory response (55).

Cytomegalovirus

CMV retinitis (Figure 5) is the most common severe ocular infection in AIDS patients. It is an AIDS-defining illness, typically occurring in patients with a CD4+ lymphocyte count below 50 cells/mm^3 (56). CMV retinitis may also occur in other immunosuppressed patients and occasionally in neonates, albeit much rarer than in late stage HIV infection. The availability of HAART has led to a significant decline in the incidence of CMV retinitis (57, 58).

Pathogenesis

The virus may be spread vertically or horizontally and is shed from multiple sites. Risk factors for severe disease include primary infection, high viral load, and immune suppression (59). CMV remains latent in immunocompetent individuals until there is an abrogation of the immune response (60).

CMV retinitis occurs following chronic CMV viremia. The virus is thought to reach the retina via infected monocytes, or, rarely, by direct extension from the CNS via the optic nerve (7). HIV microvasculopathy possibly facilitates the passage of CMV infected cells into the retina and may explain the higher incidence of CMV retinitis in AIDS patients than in patients with immunosuppression due to another cause.

Histologic examination reveals extensive retinal necrosis with little inflammatory response. Cells containing intranuclear and intracytoplasmic inclusion bodies may be found in all layers of the retina. Some of these are large with a surrounding halo, giving rise to the characteristic "owl's eye" appearance. Indeed the virus's name was derived from these cytopathic effects seen in cell culture, namely "cytomegaly" (61). Occasionally, multinucleate giant cells may be seen. CMV antigens, DNA, and virions have been found in the

FIGURE 5 Cytomegalovirus retinitis with retinal haemorrhages and exudates. Courtesy Retinal Gallery and Don Gass (http://retinagallery.com/displayimage.php?pid=3989).

nuclei and cytoplasm of infected cells using techniques such as immunohistochemistry, *in situ* hybridization and electron microscopy. Gliosis or calcium deposits mark areas of burned-out disease where retinal vessels show perivascular fibrosis and narrowing (7).

Clinical manifestations

CMV retinitis is typically painless but may be associated with mild discomfort. Symptoms will depend upon the location of lesions and upon complications, such as retinal detachment (62). CMV retinitis is bilateral in approximately half of cases. In approximately 80% of those with unilateral disease, the contralateral eye will be affected, as well, in the absence of treatment (63).

When the optic nerve head or macula is affected, vision may be markedly reduced. However, many cases are asymptomatic, requiring regular screening, particularly in those with CD4+ cell counts below 100 cells/mm^3.

The necrotic process of CMV retinitis usually starts in the periphery and slowly progresses. Untreated, the entire retina is destroyed within 6 months. Symptomatic patients may complain of blurred vision, visual field defects, or floaters. Fundoscopy may reveal large, unifocal, sometimes multifocal, areas of cheesy, yellow-white, full-thickness retinal necrosis with associated intraretinal hemorrhages (7).

Typically, the vitreous cavity, choroid, and anterior segment show little or no inflammatory reaction, although fine scattered deposits are commonly observed on the corneal endothelium (64). Other than retinitis, other ocular manifestations of CMV include anterior uveitis and corneal endotheliitis (65), as well as acute retinal necrosis (66).

Diagnosis

The differential diagnosis of hemorrhagic retinal necrosis in an HIV-positive individual includes ARN, PORN, toxoplasma retinochoroiditis, fungal infection, syphilitic retinitis, intraocular lymphoma, and HIV microvasculopathy (67). The slow progression, unifocality, characteristic appearance, and absence of intraocular inflammation aid in establishing the diagnosis of CMV retinitis. However, although the diagnosis is usually made clinically, additional laboratory testing may be necessary and includes PCR assessment of blood and ocular fluids, as well as serum antibody testing (68). In atypical cases the diagnosis may be aided by electron microscopy or PCR amplification of viral DNA obtained from an endoretinal, choroidal, or vitreous biopsy. Virus isolation from blood or urine may help confirm the diagnosis (69).

Treatment

Without treatment the disease progresses relentlessly, resulting in little or no visual function. The management of CMV retinitis involves treatment with an antiviral drug in conjunction with HAART for HIV-positive patients. Antiviral treatment may be administered systemically, as intravitreal injections, or as a long-acting intravitreal implant. Systemic antiviral agents include the nucleoside analogue ganciclovir and its prodrug, valganciclovir, which has excellent oral bioavailability and offers the benefits of an orally bioavailable drug with pharmacokinetics comparable to intravenous ganciclovir (70, 71). Both require induction doses, which are given twice a day, followed by maintenance doses, given once daily. However, some HIV positive patients on HAART, who respond well to treatment, may no longer require maintenance treatment (71–76).

Ganciclovir-resistant mutants, usually the result of a mutation in the unique long 97 (UL97) region of the genome, may emerge (77). Other available drugs for the treatment of CMV disease includes foscarnet, a pyrophosphate analogue, and the nucleotide analogue, cidofovir. Systemic treatment with ganciclovir, valganciclovir, foscarnet, or cidofovir leads to suppression of the retinitis and delays progression of disease (78). Further, it has the advantage of treating associated systemic disease and protecting the contralateral eye (7).

Local treatments usually take the form of intravitreal injections containing ganciclovir or formivirsen (no longer available in the U.S.), a synthetic antisense oligonucleotide that blocks viral mRNA translation (54). Other options include intravitreal injections with foscarnet or cidofovir or implantation of a sustained-release device delivering ganciclovir slowly (79–81). Intravitreal cidofovir may precipitate hypotony and is therefore generally avoided.

Although systemic drug toxicity is avoided, there are complications associated with repeated intraocular injections, including hemorrhage, endophthalmitis and retinal detachment (7). Intraocular injections also do not address occurrence of the frequent extraocular complications of CMV infection. Laser retinal photocoagulation has not been proven to contain the spread of retinal infection but may be of use in treating retinal breaks and localized peripheral detachments. Treatment of full-blown retinal detachments is best performed by pars plana vitrectomy, internal silicone oil tampanade, and endolaser (7).

Varicella Zoster Virus

Varicella zoster virus (VZV) is spread via respiratory droplets and is highly infectious. After primary infection the virus remains latent in one or more posterior root ganglia, usually in the trigeminal or thoracic nerves. During periods of lowered immunity the virus may reactivate and travel from the ganglia down the peripheral nerves to the skin, resulting in the typical lesions of herpes zoster, occurring typically in a dermatomal distribution (3). Damage to the eye may be due to a number of processes, including viral replication and inflammatory responses to the virus. Postherpetic neuralgia is a serious complication of herpes zoster ophthalmicus (HZO), which is more likely to occur with advanced age and severe eruptions (82). Unfortunately, postherpetic neuralgia is not prevented by early systemic antivirals or corticosteroids.

Clinical manifestations

Primary VZV infection occasionally may result in vesicular lesions on the eyelids, conjunctiva, or limbus. Lid lesions may develop into excavated ulcers that cause considerable inflammation, and VZV may rarely induce severe scleritis (83, 84). The cornea may also be involved with primary infection, which may show a punctate keratitis and marginal keratitis in the presence of limbal pustules (7). Later in the disease course, after the resolution of the exanthema, corneal involvement may still occur. In addition, bleb-like lesions in the presence of corneal edema have been described, as well as disciform keratitis, which appears to be identical to HSV keratitis (7).

Approximately 10 to 20% of all cases of herpes zoster present as HZO (85). The presentation of ophthalmic zoster can be complex due to the structure and complexity of the eye. It may involve the eyelid, conjunctiva, sclera, cornea, and iris, with iritis and keratitis being the most common complications (86). The typical erythematous, pustular rash

of HZO occurs in the distribution of the first division of the trigeminal nerve. The rash may extend from the nose and the eye to the scull vertex but does not cross the midline (Figure 6). Involvement of the nasociliary branch of the trigeminal nerve occurs commonly and correlates with ocular involvement in approximately 50% of patients with this sign (7). When this does occur, Hutchinson's sign, which is a vesicular lesion at the tip of the nose, may precede the development of HZO.

Eyelid vesicles resolve with pitting pigmentation and scarring, sometimes yielding eyelid retraction, malposition, and corneal exposure. The eyelash follicles and meibomian glands may also be damaged (7). Nodular and diffuse scleritis and keratitis may occur, sometimes months later. Damage to corneal nerves may result in corneal anesthesia.

Acute retinal necrosis has been described in older patients (87) as has PORN, which is most commonly caused by VZV, which can be diagnosed by PCR amplification of viral DNA found in fluid obtained from the anterior chamber (87, 88). Also associated with ocular VZV infection is anterior uveitis, both granulomatous and nongranulomatous (89). Cranial nerve palsies and CNS involvement are occasionally seen. Long-term sequelae include postherpetic neuralgia, permanent scarring of the eyelids, entropion, keratoconjunctivitis sicca, and neurotrophic keratopathy (7).

In congenital varicella syndrome, where mothers suffer VZV infection in the first or second trimester of pregnancy, ocular involvement may be present in the newborn.

FIGURE 6 Herpes zoster ophthalmicus. Courtesy of William Charles Caccamise and Ophthalmic Atlas Images by EyeRounds.org, The University of Iowa. Note the dermatomal distribution of the lesion and scar of Hutchinson's sign at the tip of the nose which may precede the development of herpes zoster ophthalmicus.

Treatment

The treatment of VZV infection is generally aimed at treating the symptoms, speeding up recovery, and reducing complications (7). There are currently three antivirals used for the treatment of HZO, namely acyclovir, valacyclovir, and famciclovir. Intravenous acyclovir is the drug of choice for immunosuppressed patients to prevent disseminated infection. Oral acyclovir commenced within 72 hours of onset hastens the resolution of skin lesions, reduces acute pain, and reduces the incidence of ocular complications. Valacyclovir and famciclovir are at least as effective as acyclovir in preventing ocular complications and have simpler dosing schedules because of higher oral bioavailability (90, 91). In addition, topical acyclovir has been shown to hasten the resolution of corneal epithelial disease and reduce the incidence of recurrence (92).

Epstein-Barr virus

Epstein-Barr virus (EBV) infection usually follows contact with infectious saliva, and generally infects B-cells but can also replicate in subpopulations of squamous epithelial cells (see chapter 25) (93). Previous studies have found various presentations of corneal epithelial and stromal infections associated with EBV (35). However, the most common ocular manifestation of acute mononucleosis is periorbital edema and follicular conjunctivitis (94). Posterior segment diseases have been reported but are very rare. There have also been reports of ocular lymphomas including B- and T-cell lymphomas associated with EBV (35). Seroepidemiologic studies have implicated EBV in conjunctivitis, episcleritis, keratitis, retinitis, and uveitis, with uveitis being reported in numerous case reports. Indeed, EBV has been detected in all ocular tissues except the optic nerve (94, 95, 96).

No specific treatments for EBV infections of the eye have been proven effective. Treatment is usually supportive, including lubricants, analgesia, and topical steroids in cases of uveitis. The antiviral agents acyclovir and foscarnet have been used but with limited success. Interferon-alpha has also been used to treat cases with apparent success, following a poor response to acyclovir (95).

Other Herpesviruses

The other less common herpesviruses that may affect the eye include human herpesvirus-6 (HHV-6) and human herpesvirus-8 (HHV-8), with the latter also known as Kaposi sarcoma-associated herpesvirus (see chapters 24 and 26). There have been rare reports of optic neuritis and conjunctivitis been caused by HHV-6 (61, 97). HHV-8 may cause conjunctival Kaposi sarcoma and possibly uveitis (3, 35, 98).

Adenoviruses

The ocular manifestations of adenoviruses are not strain-specific (99), and while the prevalence of milder forms of ocular disease is unclear, local epidemics of more severe infections occur episodically, including in eye clinics, where water and instrument contamination may facilitate transmission (100). These nonenveloped viruses are particularly resistant to inactivation and can survive on inanimate objects for prolonged periods (see chapter 27).

Pathogenesis

Adenoviral pathology results from a combination of viral replication and cell lysis. Histopathologically, adenoviruses produce intranuclear inclusion bodies, as well as the so-called "smudge cells", which contain large amounts of capsid

TABLE 6 Adenoviral ocular syndromes

Syndrome	Adenoviral species	Common serotypes involved
Epidemic keratoconjunctivitis	Human mastadenovirus D	8, 19, 37
Pharyngoconjunctival fever	Human mastadenovirus B	3, 4, 7, 11, 14
	Human mastadenovirus E	
Nonspecific follicular conjunctivitis	Human mastadenovirus B	3, 4, 7
	Human mastadenovirus D	
	Human mastadenovirus E	
Chronic keratoconjunctivitis	Very rare—caused by many of the serotypes mentioned above	

protein. The toxic effect of large amounts of structural protein, as well as the immune response, may also contribute to adenoviral pathology (101,102).

Clinical manifestations

Ocular manifestations of adenoviral infection usually results in acute, self-limiting disease, which may vary considerably in severity. The incubation period ranges from 2 to 14 days, and viral shedding in ocular secretions occurs up to 2 weeks after onset. Symptoms vary and include pain, lacrimation, and foreign body sensations in more severe cases. Corneal involvement and anterior uveitis may produce considerable discomfort and photophobia. Vision may be reduced in acute stages by corneal epithelial and stromal inflammation and in the latter stages, by scarring. Generally the contralateral eye also becomes infected but not as severely as the primary eye (7).

Ocular manifestations of adenoviral infection are classified into four syndromes: epidemic keratoconjunctivitis (EKC), pharyngoconjunctival fever (PCF), acute nonspecific follicular conjunctivitis (NFC), and chronic keratoconjunctivitis (CKC) (Table 6) (103). In addition, adenovirus infection has been linked to acute hemorrhagic conjunctivitis.

PCF most commonly occurs in children and is usually due to serotype 3, but serotypes 1, 4, 5, 6, 7, and 14 have also been implicated. It usually presents with fever and sore throat after an incubation period of about 12 days. Ocular involvement is usually bilateral, with burning, irritation, and photophobia. A punctate keratitis may develop, followed by corneal infiltrates (103).

EKC is most commonly associated with serotype 8 and 19, although serotypes 2, 3, 4, 7 to 11, 14, 16, and 29 have also been linked. This condition is highly contagious and is most common during autumn and winter. Keratitis occurs in the minority of cases, whereas subepithelial corneal infiltrates are the hallmark of EKC; corneal infiltrates may lead to visual loss (103, 104).

Follicular conjunctivitis is the most common ocular manifestation of adenoviral infection, which usually produces a mild conjunctivitis that is self-limiting. However, conjunctival hemorrhages may occur and range from petechiae to frank subconjunctival hemorrhage. The more severe forms may also produce exudation and membrane, or pseudomembrane, formation.

Although acute disease is self-limiting, subepithelial punctate opacities may persist for weeks to years, producing the final syndrome of ocular adenoviral disease, namely CKC, which is very rare but which may result in symptoms of ocular irritation and visual disturbance.

Diagnosis

Diagnosis requires a thorough history and examination, looking for associated systemic features, as well as a history of infectious contacts. Definitive laboratory diagnosis can be made with techniques such as virus isolation either by conventional culture or shell vial culture, and PCR assessment, with the latter being more sensitive and specific, thus facilitating a more rapid and reliable diagnosis (105–107).

Treatment

In 2015, no specific antiviral agent had yet been approved for adenoviral disease; however, brincidofovir has good *in vitro* activity with anecdotal reports of successful human therapy. At the present the most important step in the management of adenoviral conjunctivitis is prevention of transmission. Treatment is directed at symptom relief with analgesia, lubricants, and cold compressors, as well as prevention of serious sequelae. In the acute stage, antibiotic drops are often given to prevent secondary infection, and cycloplegic agents may be offered for pain relief. Generally, however, antibiotics are not indicated. Povidone-iodine drops to treat conjunctivitis are cost-effective and can be beneficial due to the broad spectrum of coverage (33).

Membranes and pseudomembranes can be managed with surgical removal and careful administration of topical steroids. Treatment of keratitis and uveitis, secondary to severe adenoviral conjunctivitis, may be managed with topical steroids but under close supervision as recurrences are possible after withdrawal of treatment (3).

Picornaviruses

Of the three genera within the *Picornaviridae* family that affect humans, species within the genus Enterovirus (See chapter 46) and Parechovirus are known to cause ocular disease. Ocular manifestations include conjunctivitis, keratoconjunctivitis, and uveitis (108–110). Enteroviral eye disease may result from direct inoculation via hand-to-eye contact, or may spread to the eye after initial replication in the gastrointestinal tract.

Various serotypes have been associated with conjunctivitis. Echovirus 7, 11, and others, as well as coxsackie B2, have been isolated from the conjunctiva in sporadic cases, with echovirus additionally reported to cause keratoconjunctivitis (110).

Acute hemorrhagic conjunctivitis (Figure 7) is caused by one of two enteroviruses, a variant of coxsackie A24 and enterovirus 70 (108). In addition, enteroviral uveitis, caused by echovirus19 and two types of echovirus 11, has been reported (109).

Parechovirus, which was previously grouped under the human enteroviruses, has recently been isolated from ocular fluids in patients with uveitis (111).

Clinical Manifestations

Ocular infection is typically bilateral, with the usual symptoms of pain, foreign body sensation, and lacrimation.

FIGURE 7 Haemorrhagic conjunctivitis. Courtesy of William Charles Caccamise and Ophthalmic Atlas Images by EyeRounds.org, The University of Iowa.

Clinical features include eyelid edema, follicular conjunctivitis, chemosis, and subconjunctival hemorrhages. Corneal involvement is limited to superficial epithelial keratitis, while bacterial superinfection has also been reported, particularly in patients treated with topical steroids (112).

Diagnosis

Diagnosis of picornaviral ocular disease is based upon clinical examination in addition to laboratory testing, such as viral isolation or PCR evaluation from conjunctival scrapings, with neutralizing antibody tests being less helpful (113).

Treatment

Ocular disease due to picornaviruses is usually self-limiting, allowing conservative management with cold compressors and lubrication. Various agents have been shown to inhibit enteroviral replication *in vitro*, but none are available as chemotherapeutic agents (103). Topical steroids should be avoided because of the risk of corneal perforation (33).

Measles

Measles virus, a member of the *Paramyxoviridae* family of single-stranded RNA viruses, is transmitted via the respiratory route and is highly contagious (114, 115). After an incubation period of 8 to 12 days, the symptoms of fever, cough, coryza, and conjunctivitis begin. The typical rash lasts for 3 to 7 days. Complications of measles may be caused by disruption of epithelial surfaces, as well as by immunosuppression (see chapter 38) (114).

The ocular manifestations of measles first appear during the prodrome and include subepithelial conjunctivitis with elevated papules (Koplik spots) (7). These may develop into epithelial keratoconjunctivitis that first appears on the exposed parts of the conjunctiva and progresses toward the centre of the cornea. Involvement of the cornea gives rise to photophobia that is characteristic of measles virus infection (7). In general, most patients will develop conjunctivitis, while keratitis occurs less often. In resource-poor countries with poor sanitation and malnutrition, particularly with vitamin A deficiency, measles may result in corneal disease with ulceration, keratomalacia, secondary bacterial infections, and corneal perforation (116). Indeed, measles virus is the most important cause of blindness in children in developing countries (115). Acute measles virus infection depresses the serum retinol concentration, which may manifest as xerophthalmia and eventually result in blindness. Blindness may also result from cortical damage from measles encephalitis (114).

Treatment of conjunctivitis is symptomatic, and recovery is usually complete and without sequelae. In poorer countries, where measles is often associated with vitamin A deficiency, keratomalacia is a severe complication and should be treated with urgency. Systemic vitamin A supplementation is required, while local lubrication, topical retinoic acid, and sometimes surgical intervention may be required (54).

Poxviruses

Molluscum contagiosum

Molluscum contagiosum virus is a fairly common viral disease, which is spread by direct contact and causes a papular eruption on skin and mucous membranes anywhere on the body (see chapter 19) (3). In patients with HIV infection, the lesions may be atypical and extensive (117) and are recognized as an ocular complication of AIDS. Such lesions are also being reported in patients undergoing systemic steroid therapy (118). The typical umbilicated nodules are most common on the trunk or in the axilla, but the periocular skin and, rarely, the conjunctiva, may be affected (Figure 8).

The virus may affect the eyelid, conjunctiva, and cornea, predominantly in young adults (119). A uniocular chronic follicular conjunctivitis is typical and represents a reaction to virus particles shed into the tear film (7).

Most lesions resolve spontaneously over weeks. Should treatment be required, such as in highly immunosuppressed patients, options include cryosurgery or curettage. In addition, cidofovir has been shown to contribute toward clearance of advanced lesions in HIV-associated disease (120).

Vaccinia Virus

With the eradication of smallpox and discontinuation of vaccination programs in the 1970s, ocular disease due to vaccinia virus is rare. However, with a recent resurgence of vaccinations, several cases of ocular vaccinia have been reported (see chapter 19) (121). The clinical manifestations are more severe in immunosuppressed patients and include ulcerating eyelid pustules, blepheroconjunctivitis, eyelid edema, and papillary conjunctivitis, while corneal involvement occurs in about 30% of cases. Although ocular complications of vaccinia virus are usually self-limiting in

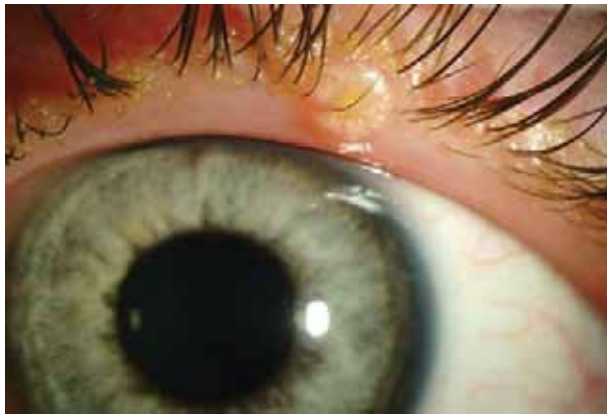

FIGURE 8 Eyelid molluscum contagiosum with typical umbilicated lesion. Courtesy Jesse Vislise and Ophthalmic Atlas Images by EyeRounds.org, The University of Iowa.

immunocompetent individuals, treatment may be required in certain cases. Options include vaccinia immune globulin, which is the only FDA approved medication for the management of complications resulting from smallpox vaccination (7, 122). Experimental agents include the nucleoside analogues cidofovir and brincidofovir, which have shown *in vitro* activity against poxviruses, with the latter having less renal toxicity and greater oral bioavailability. (123,124). Another investigational agent is the compound ST-246 (tecovirimat), which inhibits the release of infectious virus from infected cells and was shown to work in animal models (125). Other agents that have activity against vaccinia are adefovir and ribavirin, which have been considered for treatment of serious complications of smallpox vaccination. (126,127).

Human Papillomavirus

Human papillomaviruses (see chapter 29) may cause a variety of ocular epithelial disorders, including common warts on the eyelids, conjunctival papillomas in the fornices or at the limbus, conjunctival squamous cell carcinoma in associations with types 16 and 18, and non-neoplastic conditions, such as climatic droplet keratopathy (128), which is a disease characterized by accumulation of transparent material in the superficial layers of the corneal stroma (129).

Lesions occurring on the eyelids share the general histologic features of lesions occurring on other keratinizing epithelia. Eyelid lesions may be disfiguring or result in ptosis, while those on the eyelid margin may cause chronic papillary conjunctivitis or a punctate epithelial keratitis (7). While types 16 and 18 are associated with conjunctival carcinoma, types 6 and 11 cause benign papillomas (130).

Treatment of conjunctival lesions is usually by surgical excision. Adjunctive therapies include cryotherapy and cautery to the base of the lesion. Incomplete excision may result in recurrence. Recurrence or severe disease may be treated with systemic interferon or topical cytotoxic agents (7).

Ocular Disorders Associated with Systemic Viral Illness

Numerous other viruses are reported to manifest with an ocular complication as part of the spectrum of disease. Some of these occur very rarely and include influenza virus, mumps, BK virus, human T-cell leukemia virus type 1 (HTLV-1), Rift Valley fever, dengue, Lassa virus, and other arenaviruses (131), plus other viruses more commonly associated with hemorrhagic fevers, and hepatitis C virus. Many systemic viral infections are associated with conjunctival injection and retro-orbital pain.

Influenza (see chapter 43)

The H7 subtype of avian influenza virus is the predominant influenza virus associated with conjunctivitis (132, 133), although this has not been a feature of the recent avian H7N9 outbreak. The commonest influenza viruses able to infect numerous ocular cell types are the highly pathogenic avian influenza viruses H7N7 and H5N1 (3, 132). The neuraminidase inhibitor oseltamivir has recently been shown to inhibit H7N7 and H7N3 replication in the ocular tissue of mice (134) and is thus a potential treatment option.

Mumps

Most patients with mumps present with parotitis, and complications include orchitis, oophoritis, aseptic meningitis, encephalitis, pancreatitis and, very rarely, ocular complications (see chapter 39) (135). The most common form of ocular involvement is dacroadenitis, although conjunctivitis, which may be associated with subconjunctival hemorrhage, is not unusual (136). An association between mumps virus infection and keratitis and iritis has been reported previously (137). Corneal involvement is unilateral, along with painless interstitial keratitis. Visual acuity is usually decreased in the affected eye, but recovery is complete and long-term sequelae rare (3).

Human T-cell Leukemia Virus Type 1

The two important diseases caused by HTLV-1 are adult T-cell leukemia/lymphoma and HTLV-1-associated myelopathy/tropical spastic paresis (HAM/TSP), but HTLV-1 can cause uveitis (see chapter 33). The uveitis caused by HTLV-1 is characterized by granulomatous and nongranulomatous inflammation with associated vitreous opacities and retinal vasculitis in one or both eyes (138). The uveitis may occur as a primary event or may occur with HAM/TSP. Other ocular manifestations include opportunistic chorioretinal infections, lymphomatous infiltrates in patients with adult T-cell leukemia/lymphoma, retinal pigmentatory degeneration, episcleritis, keratouveitis, and keratoconjunctivitis sicca in patients with HAM/TSP (3, 139). Diagnostic laboratory tests include serology for IgG and IgM, as well as PCR of blood or ocular tissues.

BK Virus

BK virus, a member of the *Polyomaviridae* family (see chapter 28), may reactivate under conditions of immune suppression and spread to other organs (140). Reactivation of latent infection may present as an atypical retinitis (141).

Hepatitis C virus

The extrahepatic complications of hepatitis C (see chapter 54) can include ocular involvement of the cornea, conjunctiva, and accessory lacrimal glands, with dry eye syndrome being a common manifestation of chronic hepatitis C. Retinal involvement has also been reported (3, 142). Treatment of chronic hepatitis C has resulted in improvement in certain ocular hepatitis C manifestations. On the other hand, treatment with interferon and ribavirin is also associated with ocular disease, including retinopathy (143).

Viruses Associated with Hemorrhagic Fevers

Viral hemorrhagic fevers (VHF) usually present as an acute febrile illness, characterized by increased vascular permeability, malaise, and eventually prostration. Symptoms are usually due to damage to the endothelium, which commonly is associated with conjunctival injection and may manifest with ocular symptoms (144). The filoviruses, Ebola and Marburg (see chapter 42), may cause conjunctivitis as part of the disease syndrome. In addition, the convalescent phase of Ebola virus disease has recently been associated with severe unilateral uveitis, where viable Ebola virus was isolated from aqueous humor (145). In addition to conjunctivitis, arboviruses such as Rift Valley fever virus (RVFV) (see chapter 44) and dengue (see chapter 53), may affect other parts of the eye as well.

RVFV may be associated with uveitis and chorioretinitis (146), and conjunctivitis is common, sometimes associated with subconjunctival hemorrhages. Dengue fever has also been implicated in ocular disease, albeit very rarely. Retinal and conjunctival diseases due to dengue virus infection have been reported (147).

TABLE 7 Common congenital viral infections with ocular manifestations

Virus	Congenital ocular features
CMV	Retinitis, cataract, optic atrophy
Rubella	Pigmentatory retinopathy, cataract, glaucoma, microphthalmia
VZV	Optic atrophy, chorioretinitis, cataract, microphthalmia
HSV	Ophthalmia neonatorum, keratitis, cataract, microphthalmia

CMV, cytomegalovirus; VZV, varicella zoster virus; HSV, herpes simplex virus.

Chikungunya virus (see chapter 55) may uncommonly present as a viral hemorrhagic fever. In a recent retrospective review out of India, chikungunya was also associated with ocular complications, mostly iridocyclitis, retinitis, and episcleritis (148).

Viruses Associated with Congenital Ocular Disease

A variety of infectious agents result in congenital infection, some of which may manifest with an ocular complication. The viruses associated with congenital ocular disease are illustrated in Table 7. Although CMV, VZV, and HSV may rarely result in congenital ocular disease, congenital rubella (see chapter 56) is well known for producing ocular complications.

Approximately 10% of neonates with congenital CMV will have symptoms at birth (149). Of these, approximately 10% will present with chorioretinitis and/or optic atrophy, while the frequency of symptomatic infection has recently been shown to be similar for maternal primary infection versus reactivation (149,150). A previous study, however, found that ocular symptoms in congenital CMV were more common among patients born after a primary maternal infection than among those born after a recurrent maternal infection. (151).

Neonatal HSV may occur as a result of intrauterine infection (rare), perinatal infection (approximately 85%), or postnatal infection (approximately 10%). Ocular involvement most commonly consists of blepheroconjunctivitis or keratitis. (152).

Congenital Rubella

The Australian ophthalmologist, Norman Gregg, first described the teratogenic effects of the rubella virus in the 1940s (153). Other than Fuchs heterochromic iridocyclitis, the ocular manifestations are generally limited to the congenital rubella syndrome (154–156). Damage to the fetal endothelial cells within the first 12 weeks of pregnancy is the result of viral replication, since the immune system has not developed by this time (157). In addition, retardation of mitosis in infected cells contributes to the pathogenesis.

In a study from the early 1990s, ocular disorders were found to be the most common signs in patients with congenital rubella syndrome (CRS) (158). Ocular defects include cataract, glaucoma, retinopathy, microphthalmia, and iris hypoplasia. The most common of these were pigmentatory retinopathy and cataract. Cataracts may be bilateral, nuclear, lamellar, or total and may progress to complete opacity. Persistence of live virus in the lens after birth may result in the development of cataracts after birth in previously clear lenses (159).

Retinopathy is usually bilateral and affects the retinal pigment epithelium in isolation, resulting in widespread, irregular pigment deposits of variable size, most numerous at the macula (7). Glaucoma associated with CRS probably results from aberrant development of the anterior chamber angle, while chronic uveitis and an enlarged cataract lens may also contribute.

The diagnosis of CRS can be made by demonstrating rubella specific IgM synthesized by the fetus, which is present at birth. An absence of IgM in the neonatal period virtually excludes symptomatic CRS (157). Culture methods, as well as molecular methods such as PCR, may also be used for direct viral detection. Oral fluid, urine, blood, CSF, lens aspirates, and postmortem tissues are all suitable specimens (3).

Management of CRS is aimed at treating complications, such as performing cataract surgery, and the management of secondary glaucoma. For infections occurring after birth, which may manifest as conjunctivitis or keratitis, symptomatic treatment may be necessary, although the condition is usually self-limiting (33).

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Antiretroviral Agents

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11

In 1987, zidovudine became the first approved agent in the United States for the treatment of human immunodeficiency virus type 1 (HIV-1) infection. Almost 30 years later, more than 26 additional agents in six drug classes have been approved. These include nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), a fusion inhibitor (entry inhibitor), a chemokine coreceptor antagonist (entry inhibitor), integrase strand transfer inhibitors (Table 1), and pharmacokinetic enhancers. This success is the result of a prodigious effort to dissect the virus' replication cycle and the virion's interaction with its CD4 target cells to identify promising drug targets. It also illustrates the interdependency of the drug development process, knowledge of disease pathogenesis, and use of sensitive therapeutic monitoring tools like plasma HIV-1 RNA levels and drug resistance testing.

The expanding availability of fixed-dose combinations (FDCs) (Table 6) has revolutionized treatment over the past 20 years by providing high potency and excellent safety profiles, simplifying treatment, improving quality of life, and facilitating adherence to therapy (Table 1). In resource-rich settings with full formulary access to approved drugs and FDCs, the expectation is that viral suppression can be achieved in more than 90% of individuals, including those with high degrees of treatment experience and multidrug-resistant virus (1).

This chapter describes the major characteristics of antiretroviral agents that are currently approved, or at a promising stage of clinical development, and is organized according to the virus' replication cycle (Fig. 1). This chapter complements chapter 34, which discusses the virologic, pathogenetic, and clinical aspects of HIV disease and in which the principles of antiretroviral treatment and the prevention of resistance are discussed.

HIV-1 ENTRY INHIBITORS

HIV cell entry is characterized by a series of complex virus-host cell interactions that are each a potential step for inhibition. HIV entry into the CD4 cell requires nonspecific interactions with the cell surface heparin sulfates, followed by highly specific binding to the CD4 receptor and either the CCR5 or CXCR4 coreceptor, leading to conformational change in the envelope transmembrane subunit, and finally, virus-host cell membrane fusion (2–4). Both viral envelope (gp120 and gp41) and cellular receptors (CD4 and CCR5/CXCR4) are involved in HIV fusion and entry and are attractive targets for drug development (Fig. 1) (5). Currently, two drugs are approved in this class with several others in clinical development. Selected investigational agents are summarized in Table 2.

Enfuvirtide

Enfuvirtide (Fuzeon, T-20; formerly DP-178, pentafuside) is a membrane fusion inhibitor that interferes with HIV gp41 protein-mediated virus-cell fusion (Figs. 1 and 2). The agent is a 36-amino-acid synthetic peptide that is derived from the second heptad repeat (HR2) of HIV-1 gp41. This molecule interacts with sequences within the first heptad repeat (HR1) of the pre-hairpin intermediate, thereby perturbing the transition of gp41 into an active state (3, 23).

Enfuvirtide's 50% inhibitory concentrations (IC_{50}) for wild-type strains average 1.7 ng/ml. Several *in vitro* studies have suggested that susceptibility to enfuvirtide may vary by coreceptor usage, but virologic and clinical outcomes do not differ by coreceptor specificity (23–25). Enfuvirtide inhibits a broad range of non-clade B viruses and strains resistant to other classes of antiretroviral agents but has no activity against HIV-2, exhibiting additive to synergistic activity when combined with members of other antiretroviral drug classes. Although enfuvirtide is a gp41 derivative, its pharmacokinetic and antiviral effects are not affected by preexisting or developing anti-gp41 antibodies.

Pharmacology

The low oral bioavailability of enfuvirtide necessitates parenteral administration. It is available in powder form and must be reconstituted with sterile water before administration by subcutaneous injection. The current dosage in adults is 90 mg twice daily (b.i.d.) administered subcutaneously into the arm, thigh, or abdomen with all sites having comparable pharmacokinetics. The bioavailability of the 90-mg dose is about 84% with a mean steady-state trough level of 3.3 μg/ml and a mean elimination half-life ($t_{1/2}$) of 3.8 h (1.8 h after intravenous dosing). The drug is approximately 92% protein bound. No dose adjustments are required in the setting of hepatic or renal impairment.

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TABLE 1 Commonly used antiretroviral agents: indications and dosing regimens

Antiretroviral agent (year approved)	HIV-1	HIV-2	HBV[a]	Standard adult dosing for HIV
Fusion and entry inhibitors				
Enfuvirtide (2003)	✓			90 mg (1 ml) s.c. twice daily[b]
Maraviroc (2007)	✓	✓		300 mg orally twice daily
				Dose adjustments:
				• 150 mg orally twice daily with CYP3A inhibitors
				• 600 mg orally twice daily with CYP3A inducers
NRTIs				
Zidovudine (1987)	✓	✓		300 mg orally twice daily
Lamivudine (1995)	✓	✓	✓	300 mg orally once daily or 150 mg orally twice daily[c]
Abacavir (1998)	✓	✓		600 mg orally once daily or 300 mg orally twice daily
Emtricitabine (2003)	✓	✓	✓	200 mg orally once daily[d]
Tenofovir disoproxil fumarate (2001)	✓	✓	✓	300 mg orally once daily
Tenofovir alafenamide (2015)	✓	✓		25 mg orally once daily[e]
NNRTIs				
Nevirapine (1996)	✓			Initial: 200 mg orally once daily for 2 weeks
				Maintenance: 400 mg once daily extended release or 200 mg orally twice daily immediate release
Efavirenz (1998)	✓			600 mg orally once daily in the evening[f]
Etravirine (2008)	✓			200 mg orally twice daily
Rilpivirine (2011)	✓			25 mg orally once daily[g]
INSTIs				
Raltegravir (2007)	✓	✓		400 mg orally twice daily
Dolutegravir (2013)	✓	✓		50 mg once daily[h]
Elvitegravir (2014)	✓	✓		150 mg once daily with cobicistat[i]
PIs[j]				
Lopinavir/ritonavir (2000)	✓	✓		400 mg/100 mg orally twice daily
Tipranavir (2005)	✓	✓		500 mg orally twice daily
Atazanavir (2003)	✓	✓		300 mg once daily with pharmacokinetic booster
				400 mg once daily without booster for ART naïve only
Darunavir (2006)	✓	✓		800 mg orally once daily
				≥1 darunavir-resistance associated mutations: 600 mg twice daily
Pharmacoenhancers				
Ritonavir (1996)	✓	✓		100–400 mg orally per day as a pharmacokinetic booster
Cobicistat (2014)				150 mg once daily with appropriate PI or INSTIs

[a]Tenofovir alafenamide has activity against HBV, but is not FDA approved to treat HBV. Emtricitabine is approved for HBV treatment in combination with tenofovir disoproxil fumarate. Please see chapter 13 for comprehensive information on dosing for HBV disease. Dosing presented in this table is for HIV alone.
[b]s.c., subcutaneously.
[c]Lamivudine dosing for HBV disease without HIV infection is 100 mg once daily, see chapter 13.
[d]Alternative emtricitabine oral solution: 240 mg (24 ml) once daily.
[e]Tenofovir alafenamide is currently only available for those with CrCl ≥30 ml/minute in combination with emtricitabine, or as a single-dose formulation with elvitegravir, cobicistat, and emtricitabine.
[f]Efavirenz dose adjustments required if given with rifampin or voriconazole.
[g]Rilpivirine must be taken with a normal to high-calorie meal (at least 533 calories) to ensure absorption and cannot be given with proton pump inhibitors.
[h]Dolutegravir is given 50 mg twice daily for INSTI-experienced patients with certain INSTI-associated resistance mutations and when coadministered with carbamazepine, efavirenz, fosamprenavir/ritonavir, tipranavir/ritonavir, or rifampin.
[i]Elvitegravir dosing is 150 mg once daily if given with cobicistat, darunavir/ritonavir, fosamprenavir/ritonavir, or tipranavir/ritonavir. Dosing is 85 mg once daily if given with atazanavir/ritonavir or lopinavir/ritonavir.
[j]All protease inhibitors must be given with a pharmacoenhancer, either ritonavir or cobicistat, unless otherwise noted.

Adverse Effects

Adverse effects include local inflammation around the injection site, which may be related to its action as a phagocyte chemoattractant and a chemotactic agonist via the phagocyte *N*-formyl peptide receptor (26). Painful, erythematous, indurated nodules have been reported to occur in as many as 98% of individuals participating in clinical studies, but this uncommonly led to drug discontinuation (7% of subjects) or local infection (1.7%) in clinical trials. The majority of individuals experience their first injection site reaction during the initial week of treatment. Bacterial pneumonia has been reported to occur with greater frequency among patients on enfuvirtide. Risk factors for pneumonia include low initial CD4 cell count, high initial viral load, intravenous drug use,

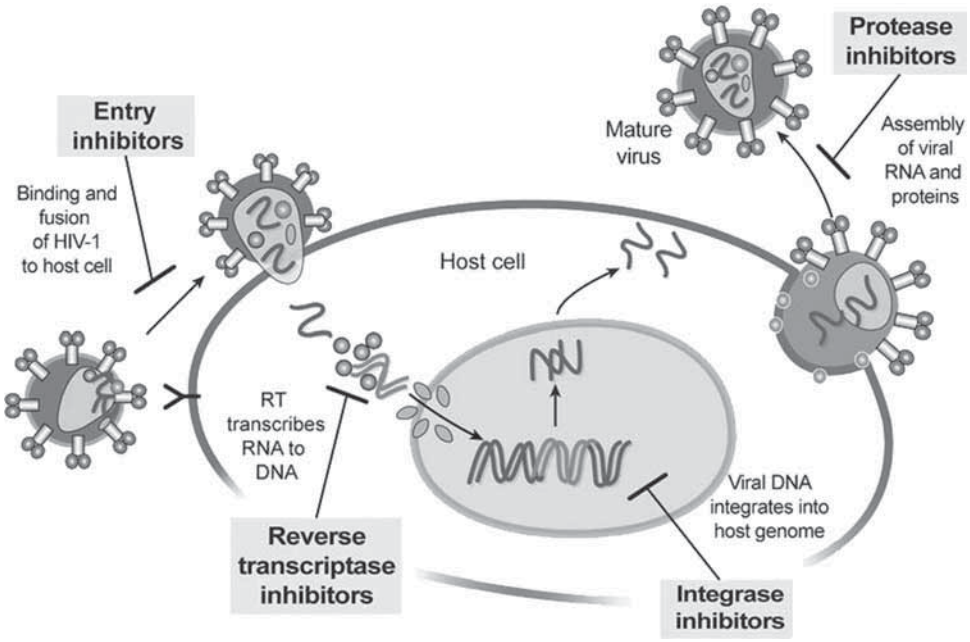

FIGURE 1. Replication cycle of HIV-1 with current targets for antiretroviral therapy.

smoking, and a history of lung disease, but direct causality has not been proven (27). Laboratory abnormalities include development of eosinophilia in 10.1%.

Drug Interactions

Enfuvirtide is not an inhibitor of cytochrome P450 (CYP450) enzymes. No clinically significant interactions with other antiretroviral agents have been identified to date.

Resistance

Resistance to enfuvirtide *in vitro* is associated with amino acid mutations at positions 36 to 43 within the first heptad repeat (HR1) target domain of gp41 (28). These changes confer alterations in HR1 binding affinities and corresponding increases, up to 34-fold, in the IC_{50} (29). Enfuvirtide has a low genetic barrier to resistance, and resistant variants appear within 2 to 4 weeks during monotherapy. In patients with early treatment failure, the most common amino acid mutations are G36D/S and V38A/M/E; mutations at codons 40 (Q40H) and 43 (N43D) seem to emerge more slowly than those at codons 36 and 38 (30). Other mutations associated with enfuvirtide resistance emerging during treatment include I37V, Q39R, and N42T (31, 32). These findings highlight the importance of combining enfuvirtide with other active agents to ensure the probability of virologic suppression. In growth competition assays, enfuvirtide-resistant isolates containing I37T, V38M, or G36S/V38M HR1 domain mutations appear to be less fit (33). The N43D single mutation confers decreased fitness for which the E137K mutation can compensate.

These mutations in the envelope gene do not appear to diminish the susceptibility of HIV-1 to other classes of viral entry inhibitors, including coreceptor (CCR5 and CXCR4) antagonists (34). In patients with virologic failure, changes other than those described for the HR1 region may contribute to the poor response (24), such as mutations or polymorphisms in the HR2 region, as well as CCR5 coreceptor usage and density (35). Increased affinity for the gp120 coreceptor is also associated with increased resistance to both enfuvirtide and maraviroc (a CCR5 antagonist) (36).

Clinical Applications

In treatment naïve individuals, enfuvirtide, when given as an intensification of combination therapy, neither improves the immunologic response nor reduces the latent viral reservoir (37, 38). Enfuvirtide has proven beneficial, however, in highly treatment-experienced patients when treatment options are limited. Studies of patients with preexisting multidrug class resistance have established that (i) addition of enfuvirtide to an optimized background significantly improves rates of viral suppression to <400 copies/ml and results in greater CD4 cell increases (39, 40); (ii) enfuvirtide-naïve patients who add enfuvirtide to the protease inhibitors, darunavir-ritonavir or tipranavir-ritonavir, and with raltegravir and maraviroc achieve better rates of virologic suppression to <50 copies of HIV-1 RNA/ml than patients whose optimized background did not include enfuvirtide (41, 42). The greatest benefit of enfuvirtide has been seen in patients with CD4 cell counts of >100 cells/mm^3, plasma HIV-1 RNA levels $<100{,}000$ copies/ml, and at least two active drugs in the background regimen (43). The week 12 virologic and immunologic responses to enfuvirtide are highly predictive of subsequent response (44). Advances in antiretroviral therapy have marginalized the use of enfuvirtide, which is now essentially reserved for rare, difficult multidrug-resistant cases for which a suppressive oral regimen cannot be devised. Much was learned about the molecular mechanisms of HIV entry during enfuvirtide's development, however.

CCR5 Antagonists

The rationale for development of CCR5 antagonists in the treatment of HIV-1 infection was based on the observations that (i) R5 HIV-1 uses the CCR5 chemokine receptor for entry into human cells and (ii) a 32-bp deletion in the CCR5 coding region confers natural resistance to infection

TABLE 2 Selected investigational agents

Agent	Mechanism of action	Study phase	Comments
Fostemsavir (BMS-663068)	Attachment inhibitor	III	Attaches to gp140. Prodrug of temsavir. Active regardless of tropism. Phase IIb study compared to atazanavir/ritonavir showed similar response rates at 48 weeks (6, 7).
Cenicriviroc (CVC, TAK-652, TBR-652)	CCR5 Inhibitor	IIb	Dual inhibitor of CCR2 and CCR5. Similar viral suppression rates for patients taking 100 mg cenicriviroc (76%), 200 mg cenicriviroc (73%), and with efavirenz (71%) (8).
Ibalizumab (Hu5A8, TMB-355, TNX-355)	Entry inhibitor	III	Humanized monoclonal antibody directed at CD4 receptor. In a 24-week study of ibalizumab plus optimized background as a bi-monthly intravenous injection in treatment-experienced patients, 82.5% of patients met the primary endpoint of ≥ 0.5 $\log_{10}$ decrease in viral load at 7 days (9). Subcutaneous and intramuscular injection dosage forms under development.
PRO 140 (PA14)	Entry inhibitor	IIb / III	Humanized IgG4 monoclonal antibody directed against CCR5. Studied as subcutaneous injections. A phase IIb study as single-agent maintenance therapy has maintained virologic suppression for 1.5 years in some patients (10).
Sifuvirtide	Entry inhibitor	II	Peptide that is more efficient in inhibiting HIV fusion compared to enfuvirtide (11).
HGS101	Entry inhibitor	Preclinical / I	Monoclonal antibody directed at CCR5. Derivative of HGS004 that shares the same characteristics, but has 5 to 10 times the potency of the original drug HGS004. Maraviroc more active against resistant HIV-1 and more potent against sensitive HIV-1 when combined with HGS004 or HGS101 (12).
Doravirine (MK-1439)	Reverse transcriptase inhibitor	III	NNRTI. *In vitro* activity against virus with K103N and Y181C mutations. In a phase IIb study, overall treatment response at 48-weeks comparable in those treated with doravirine and those taking efavirenz (76% vs. 71%, respectively) with less drug-related adverse events including CNS adverse events in those treated with doravirine (13). Doravirine primarily metabolized by CYP3A4 without inhibitory or inductive potential.
Dapivirine (TMC120)	Reverse transcriptase inhibitor	III	NNRTI. Intravaginal ring being developed as a pre-exposure prophylaxis strategy. Reduced the incidence if HIV-1 infection by 27% compared to placebo with further reductions in patients with increased adherence (14).
Censavudine (Festinavir, BMS-986001, OBP-601)	Reverse transcriptase inhibitor	II	NRTI. Thymidine analog (derivative of stavudine) more potent against HIV-2 than HIV-1 but with potentially less toxicity than stavudine (15). Viruses with thymidine analogue mutation (TAM) pattern 1 and pattern 2 have reduced susceptibility to censavudine.
MK-8591	Reverse transcriptase inhibitor	I	Long-acting NRTI with the triphosphate form having a half-life of 150–160 h in human PMBCs. Single 10 mg dose in naïve patients resulted in a mean viral load reduction of 1.67 $\log_{10}$ at 168 h post-dose that declined thru day 10 (16). Potential for weekly oral dosing and long-acting parenteral formulation dosed every 6 months or longer.
KM-23 (KM-023)	Reverse transcriptase inhibitor	I	NNRTI. KM-023 has demonstrated dose- and time-dependent nonlinear pharmacokinetic properties over a dose range (75–600 mg) in healthy subjects (17).
Bictegravir (GS-9883)	Integrase strand transfer inhibitor	III	Part of fixed-dose combination product containing bictegravir/emtricitabine/tenofovir alafenamide. Improved resistance profile compared to other integrase inhibitors.

(*Continued*)

TABLE 2 Selected investigational agents (*Continued*)

Agent	Mechanism of action	Study phase	Comments
Cabotegravir (S/GSK1265744)	Integrase strand transfer inhibitor	IIb	Studied as a long-acting (half-life 21–50 days) parenteral nanosuspension administered via intramuscular or subcutaneous injection and a shorter half-life (40 hours) oral dosage form. Oral cabotegravir effective as a two-drug maintenance therapy with rilpivirine from weeks 24 to 72 compared to efavirenz plus two NRTIs (68–84% vs. 63% virologic suppression at 96 weeks, respectively) (18). Intramuscular long-acting cabotegravir plus rilpivirine every 4 weeks or every 8 weeks as maintenance therapy in patients with undetectable viral loads was as effective as oral agents at 32 weeks (94–95% vs. 91% maintained viral suppression, respectively) (19).
BMS-955176	Maturation inhibitor	II	Second-generation maturation inhibitor. Activity maintained against reverse transcriptase, protease, and integrase inhibitor-resistant viruses, with EC_{50}s similar to those for the wild-type virus (20).
GSK 2838232	Maturation inhibitor	I	IC_{50} of 0.8–4.3 nM against a broad spectrum of 26 isolates covering a range of genotypes. Antiviral activity similar in subjects with wild-type HIV-1 or HIV-1 with Gag polymorphisms (V362, Q369, V370, and T371). Antiviral response increased with doses up to 40 mg daily, with a plateau of about 1.64 $\log_{10}$ c/ml observed at 40–120 mg daily (21).
GS-9620	Toll-like receptor-7 (TLR-7) agonist	I	Oral toll-like receptor agonist. In a placebo-controlled study in SIV-infected rhesus macaques, the use of GS-9620 after viral suppression induced transient and variable increases in plasma SIV RNAs and found significant reductions of virus in multiple tissues (22).

CCR: C-C motif chemokine receptor; NRTI: nucleoside reverse transcriptase inhibitor; NNRTI: non-nucleoside reverse transcriptase inhibitor; PMBC: peripheral blood mononuclear cell.

with R5 virus; and (iii) individuals failing to express the receptor are otherwise well (45–48). The current therapeutic niche of CCR5 inhibitors is as one component of combination regimens for patients with multidrug-resistant R5 virus. These agents are not active against HIV-1 strains that use CXCR4 (49).

Maraviroc

Maraviroc {4,4-difluoro-N-[(1S)-3-[(1S,5R)-3-(3-methyl-5-propan-2-yl-1,2,4-triazol-4-yl)-8-azabicyclo[3.2.1]octan-8-yl]-1-phenylpropyl]cyclohexane-1-carboxamide; Selzentry, UK-427,857} (Figs. 1 and 2) is a selective, slowly reversible antagonist that blocks the interaction between HIV-1 gp120 and the chemokine receptor CCR5 on host cells (50), thereby preventing gp41 from inserting the fusion peptide into the host cell membrane. Maraviroc is only active against CCR5-tropic virus, and patients should be screened for their virus coreceptor tropism before initiating treatment with maraviroc. Maraviroc is the only antiretroviral drug of its class yet approved. *In vitro*, maraviroc demonstrates no antagonism with existing antiretroviral agents and additive or synergistic activity in combination with enfuvirtide.

Pharmacology

Formulations of maraviroc include 150- and 300-mg film-coated tablets. The recommended dosage is 150, 300, or 600 mg b.i.d., depending on the CYP3A inhibitory and induction potential of concomitant therapy. The bioavailability of the 100-mg dose is 23%, and that of the 300-mg dose is predicted to be 33%. Coadministration of this agent with a high-fat meal reduces its area under the concentration-time curve (AUC) by 33%, although in clinical trials the antiviral effects of maraviroc have not been affected by food. It can therefore be administered without food restrictions. Approximately 76% of the drug is protein bound, and it has a volume of distribution of approximately 194 liters. The plasma elimination $t_{1/2}$ of the 300 mg b.i.d. dosage regimen is around 14–18 h. It is primarily excreted via feces, with only a limited amount of renal excretion (25%). Drug concentrations and associated adverse effects may be increased in patients with either renal impairment [Creatinine clearance (CrCl) <50 ml/min] or concomitant administration of CYP3A inhibitors (51).

Adverse Effects

Hepatotoxicity has been reported with maraviroc. Evidence of a systemic allergic reaction (e.g., pruritic rash, eosinophilia, or elevated immunoglobulin E) may occur before the development of hepatotoxicity. The safety of this agent has not been adequately studied in patients with significant underlying liver disease, including concomitant hepatitis B or C virus infection, and caution is advised in such patients. Five-year clinical trial follow-up data suggest hepatic events are uncommon (52). An increase in cardiovascular events

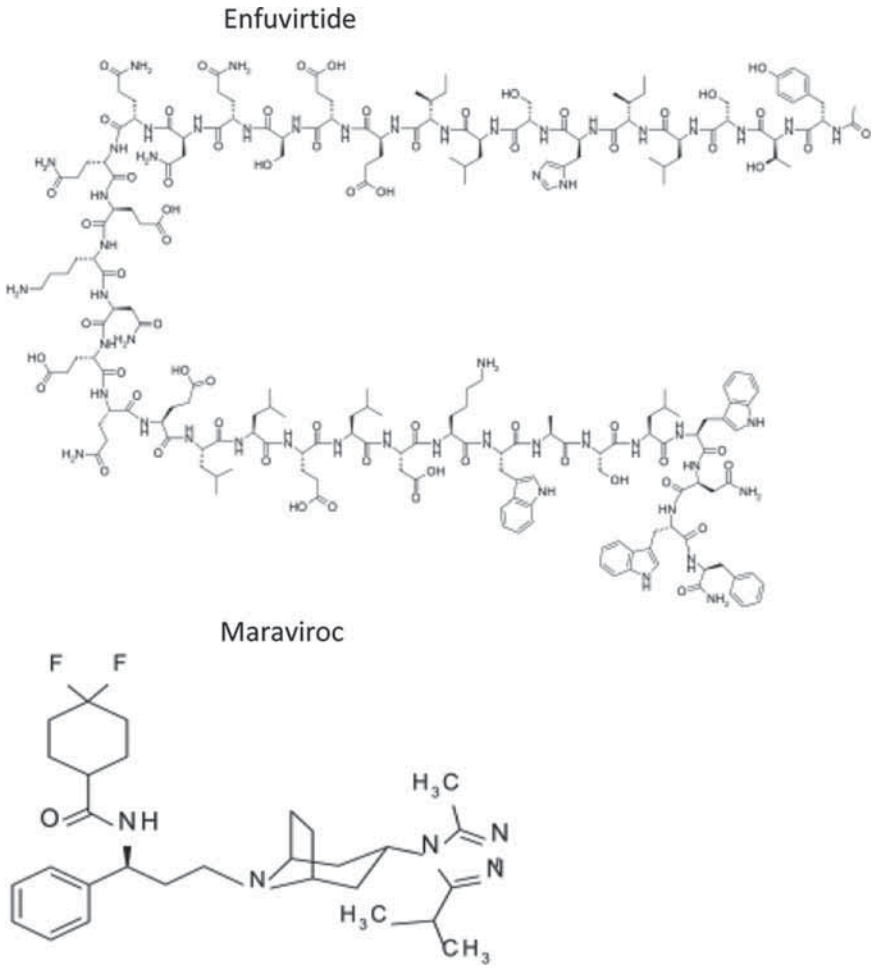

FIGURE 2. Chemical structures of HIV entry inhibitors.

(1.3%) was seen in clinical trials compared with placebo. Other more common adverse events reported were cough, pyrexia, upper respiratory tract infections, rash, musculoskeletal symptoms, abdominal pain, and dizziness, but these were infrequently associated with the need to discontinue maraviroc. Maraviroc does not adversely affect lipid profiles.

The consequences of targeting a CCR5 coreceptor on host defenses, particularly in the setting of underlying immunodeficiency, remain uncertain (47). The possibility that pharmacological blockade might increase the risk of opportunistic events, including malignancies, has not been substantiated in long-term safety data to date (52). An increase in susceptibility to severe West Nile virus infection and tick-borne encephalitis in European populations is a concern with pharmacological blockade of CCR5, given the reports of this complication in individuals who do not express an intact CCR5 coreceptor (53, 54).

Drug Interactions

Maraviroc is metabolized by CYP3A, so inhibitors of CYP3A (e.g., ketoconazole, lopinavir-ritonavir, ritonavir, atazanavir, clarithromycin) increase the concentrations of maraviroc in plasma, while CYP3A inducers (e.g., efavirenz, rifampin, phenytoin, St. John's wort) decrease maraviroc exposure. Maraviroc is not an inhibitor or inducer and will not cause clinically significant changes in concentrations of other drugs metabolized by this route.

Resistance

Two mechanisms of resistance to maraviroc have been described. Individuals with a subpopulation of CXCR4-tropic virus at baseline demonstrate a reduced virologic response to maraviroc (55). CXCR4- or dually tropic virus may emerge from a preexisting reservoir of X4 virus not detected before the initiation of treatment (56). The primary mechanism of *de novo* resistance in clinical isolates derived from maraviroc-treated patients is not related to conversion of R5 to X4 viruses *per se* although outgrowth of preexistent X4 populations can occur. Rather, R5 virus can acquire mutations that allow it to utilize the CCR5 coreceptor even in the presence of bound drug (57). Two or three amino acid mutations in the V3 loop region of the gp120 envelope allow the protein to interact with CCR5 in the drug-bound conformation and are the most likely cause of the resistance phenotype. The IC_{50} may not shift with maraviroc resistance; rather, a subset of the virus population attaches to the drug-bound coreceptor, resulting in a flattening of the high end of the susceptibility curve (57). Examining the entire susceptibility curve and reporting IC_{90} is more helpful in this circumstance.

Clinical Applications

Maraviroc is approved as part of combination treatment of HIV-1 infected patients with pure CCR5-tropic virus. However, it is not recommended as first-line treatment because it requires twice-daily dosing and does not have virologic benefits equivalent to other regimens. A trial comparing maraviroc and efavirenz-based therapy for treatment naïve patients with CCR5-tropic virus was discontinued early for not meeting prescribed efficacy criteria (58). *Post hoc* efficacy analysis at 48 weeks demonstrated noninferiority to efavirenz in those patients with CCR5-tropic virus confirmed using a more sensitive tropism assay (59), and long-term safety and efficacy were comparable to efavirenz at five years (60).

In treatment-experienced patients, two large efficacy trials have demonstrated significantly higher rates of virologic suppression to HIV-1 RNA levels of < 50 copies/ml and higher mean increases in CD4 cell counts in the maraviroc group than in the placebo group. Among patients who experienced virologic failure on maraviroc, the majority harbored X4 virus at baseline. It is therefore important to use the most sensitive tropism assay available to the clinician when contemplating use of maraviroc.

CXCR4-tropic or dually tropic virus emerges more frequently in patients with disease progression and with antiretroviral treatment experience (61). Patients infected with CXCR4-tropic, mixed-tropic, or dually tropic virus do not derive benefit from the addition of maraviroc to their regimen as do those with pure R5 virus. Of note, 44% of screened patients in the major efficacy trials harbored CXCR4-tropic or dually/mixed-tropic virus, and among treatment-experienced patients or those with first-line regimen failure, the prevalence of dually tropic virus is even higher (25, 62).

Maraviroc has also been tested in preexposure prophylaxis in HIV-negative persons (63). In a study of 406 men who have sex with men (MSM), maraviroc was shown to be safe and well tolerated in a double-blind, placebo-controlled trial which compared maraviroc, maraviroc-tenofovir disoproxil fumarate (TDF), and emtricitabine/TDF. No judgment could be made regarding efficacy since the study was not powered to detect differences in HIV-1 acquisition. The viral isolates from five seroconverters were R5 and drug-susceptible.

INVESTIGATIONAL HIV-1 ENTRY INHIBITORS

A number of investigational agents are in various stages of clinical and preclinical testing in 2016 (Table 2). They include two promising agents in later stages of development, (i) an attachment inhibitor (fostemsavir; BMS-663068) which blocks the gp120-CD4 interaction, and (ii) a humanized anti-CD4 monoclonal antibody (ibalizumab; TNX-355; TMB-355). In phase 2 testing, fostemsavir (in combination with raltegravir and TDF) yielded a similar virologic response rate at 24 weeks (61–82%) compared to ritonavir-boosted atazanavir (71%; with the same background regimen) with fewer adverse effects. In treatment-experienced patients in combination with optimized background therapy, intravenous ibalizumab resulted in as much as a 0.96 $\log_{10}$ reduction in the HIV-1 RNA level at 48 weeks. It is in phase 3 testing and can be obtained through an expanded access program (ClinicalTrials.gov NCT02707861); (iii) PRO-140, a humanized monoclonal antibody directed at the CCR5 coreceptor which has shown antiviral activity (1.0–1.5 $\log_{10}$ reductions in plasma HIV-1 RNA levels) and safety in human trials to date (64–70).

BROADLY NEUTRALIZING MONOCLONAL ANTIBODIES

HIV-infected individuals with chronic antigen exposure may produce highly potent, broadly neutralizing, cross-subtype antibodies through somatic hypermutation (71, 72). These antibodies are of great interest in preventive vaccine research. The technology for isolating these antibodies has progressed, and dozens of these broadly neutralizing monoclonal antibodies (bnAbs) have been characterized. A fraction of these have reached clinical testing for pharmacokinetics, safety, and *in vivo* anti-HIV activity (73–81). Combinations of bnAbs, bispecific bnAbs, and bnAbs in combination with long-acting antiretroviral agents (e.g., cabotegravir, rilpivirine-LA), are also under consideration (82–84). One bnAb, VRC01, directed at the CD4 binding site, has recently entered phase 2b, efficacy testing in persons at risk for HIV-1 acquisition in the Americas and southern Africa (85). For further discussion of HIV monoclonal antibodies in the context of vaccine development, please see chapter 34.

NUCLEOSIDE ANALOG REVERSE TRANSCRIPTASE INHIBITORS

Reverse transcriptase catalyzes the conversion of single-stranded genomic viral RNA into double-stranded DNA with duplicated long terminal repeats, which is subsequently integrated into cellular DNA by the viral integrase enzyme. The mature p66/p51 heterodimeric reverse transcriptase is generated by the viral protease from a homodimer by cleavage of the C-terminal RNase H domain during maturation of the viral particle. The polymerase and RNase H catalytic sites are located on p66, while p51 plays a structural role. Nucleoside analogs require intracellular triphosphorylation by host cell enzymes before incorporation by reverse transcriptase into the growing chain of viral DNA. All the currently approved nucleoside analogs target the polymerase activity of the reverse transcriptase. As with the other nucleoside analogs, the HIV-1-inhibitory concentrations of NRTIs *in vitro* depend on a number of factors, including the assay system, cell type, and virus isolate used. The pharmacology and adverse effects of these agents are summarized in Table 3.

As more potent, less toxic, and simpler regimens have moved up the priority list, others have either been discontinued or are rarely used. For agents in this category (e.g., zalcitabine, stavudine, didanosine) please see the previous edition of this chapter. An exception is being made for zidovudine given its historic significance and the fact that it is still in use globally.

Zidovudine

Zidovudine (3′-azido-3′-deoxythymidine; azidothymidine, Retrovir) (Figs. 1 and 3; Tables 1 and 3) is a synthetic thymidine analog with an azido group substituting for the 3′-hydroxyl group on the ribose ring. In peripheral blood lymphocytes, zidovudine has an IC_{50} for HIV-1, which ranges between 3 and 13 μg/ml. It has *in vitro* inhibitory activity against other retroviruses, including HIV-2 and human T lymphotropic virus type 1 (HTLV-1).

TABLE 3 Commonly used reverse transcriptase inhibitors: pharmacokinetic, major toxicity, and drug interaction characteristics

Agent	Oral bioavailability	Effect of food on AUC	$t_{1/2}$ (intracellular $t_{1/2}$), h	Major route of elimination	Adjust for renal insufficiency	Major toxicities	Major drug interactions
Zidovudine	64%	Modest decrease	1 (3)	Major inactive metabolite, G-zidovudine is renally excreted	Yes	Neutropenia, anemia, myopathy, nausea, headache, lactic acidosis, and hepatic steatosis (rare)	Myelosuppressive agents (e.g., ganciclovir): increased risk of neutropenia, anemia Rifampin, rifabutin: decreased zidovudine levels Probenecid, valproic acid, atovaquone, fluconazole, phenytoin, methadone: increased zidovudine levels
Lamivudine	86%	No	3–4 (10.5–15.5)	Mainly renally excreted unchanged	Yes	Peripheral neuropathy and pancreatitis in pediatric patients	No known significant interactions
Abacavir	83%	No	1.2 (20.9)	Metabolized by alcohol dehydrogenase and glucuronyl transferase to inactive forms which are then mainly renally excreted	No	Potentially fatal hypersensitivity reaction in 3% of cases. Asthenia, abdominal pain, headache, diarrhea, dyspepsia, and transaminitis are also reported	Methadone: decreased levels by abacavir and decreased abacavir levels
Emtricitabine	93%	No	10 (>20)	Renally excreted	Yes	Headache, nausea, insomnia; lactic acidosis and hepatomegaly with steatosis (rare), hyperpigmentation of palms and soles	No known significant interactions
Tenofovir disoproxil fumarate	>25%	Increase	>12	Mainly renally excreted unchanged	Yes	Neutropenia, headache, fatigue, renal insufficiency, bone density loss	May decrease levels of atazanavir; administer 300 mg of atazanavir with 100 mg of ritonavir when used as part of a tenofovir-containing regimen
Tenofovir alafenamide	unknown	Absorption increased >85% with high fat meal	0.5 (150-180)	CatA in PBMCs, then hepatic (carboxyesterase 1)	No, not recommended for CrCl <30ml/min	Nausea, less decrease in renal function and bone mineral density	Tipranavir-ritonavir, carbamazepine, oxcarbazepine, phenobarbital, phenytoin, rifamycins, St. John's wort: decreased tenofovir alafenamide levels Ritonavir, cobicistat: increased tenofovir alafenamide levels

(*Continued*)

TABLE 3 Commonly used reverse transcriptase inhibitors: pharmacokinetic, major toxicity, and drug interaction characteristics (*Continued*)

Agent	Oral bioavailability	Effect of food on AUC	$t_{1/2}$ (intracellular $t_{1/2}$), h	Major route of elimination	Adjust for renal insufficiency	Major toxicities	Major drug interactions
Nevirapine	>90%	Not available	>24	Hepatic (CYP450 system)	No	Rash, including Stevens-Johnson syndrome; fever; myalgias; hepatic toxicity, including acute hepatic failure	Etravirine, methadone: decreased levels by nevirapine Rifabutin, rifampin: decreased nevirapine levels Fluconazole: increased nevirapine levels
Efavirenz	40–50%	A high-fat meal increases the oral bioavailability in humans by 50%	40–55	Hepatic enzymes CYP3A and CYP2D6	No	Rash, CNS symptoms (e.g., dizziness, abnormal dreams), hepatotoxicity	Lopinavir/ritonavir, atorvastatin, carbamazepine, diltiazem, methadone, rifabutin, simvastatin: decreased levels by efavirenz Carbamazepine, phenobarbital, phenytoin, rifampin: decreased efavirenz levels
Etravirine	Unknown	Food increases oral bioavailability; should be taken following a meal	41	Hepatic (CYP450 system)	No	Nausea, rash	Efavirenz, clarithromycin, rifabutin: decreased levels by etravirine Clarithromycin, fluconazole, voriconazole: increased etravirine levels Lopinavir/ritonavir, ritonavir, nevirapine, tipranavir, rifabutin, rifampin: decreased etravirine levels

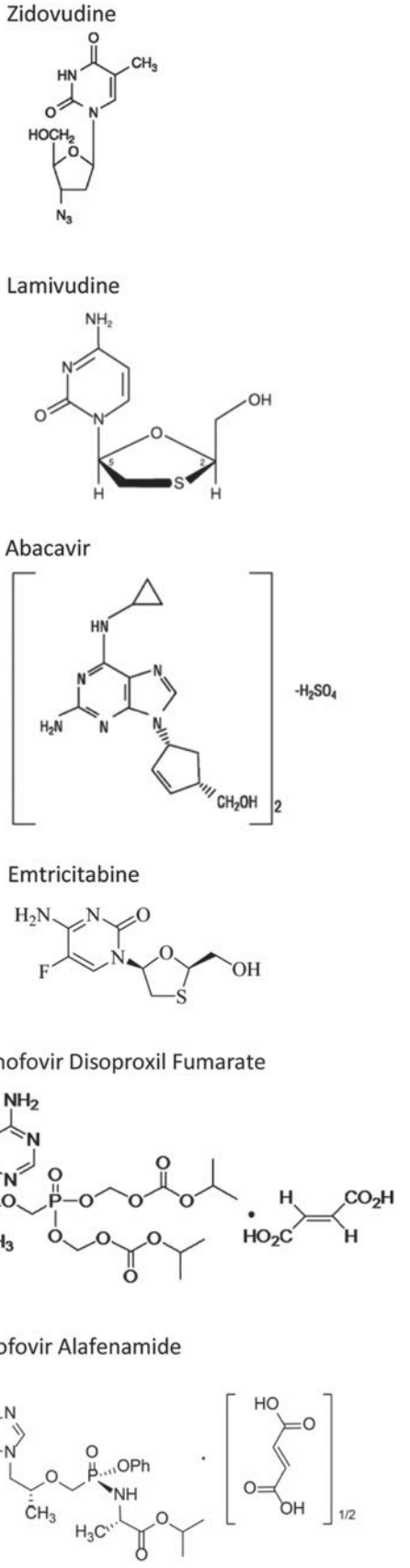

FIGURE 3. Chemical structures of HIV nucleoside/nucleotide reverse transcriptase inhibitors.

Mechanism of Action

The antiviral activity of zidovudine is mediated by its intracellular 5′-triphosphate derivative. The mono- and diphosphate forms of zidovudine are generated by cellular thymidine and thymidylate kinases, respectively. The formation of zidovudine diphosphate appears to be a rate-limiting step, as reflected by higher intracellular zidovudine monophosphate levels due to inhibition of thymidylate kinase. The final phosphorylation is completed by a cellular nucleoside diphosphate kinase. Zidovudine triphosphate then acts as a competitive substrate for the HIV reverse transcriptase and is incorporated into the elongating 3′ end of the yet-unintegrated proviral DNA. This results in the premature termination of chain elongation due to the inability of the nucleoside analog to form a normal 3′–5′ phosphodiester linkage.

Zidovudine and the other nucleoside analogs often vary in their antiviral activities across different primary cell types *in vitro*. Zidovudine is more potent in activated cells than nonactivated cells. The thymidine kinase required for zidovudine phosphorylation is an S-phase-specific enzyme and thus has increased activity in stimulated cells and lowered activity in resting cells.

Pharmacology

Zidovudine is available in capsule, syrup, and intravenous formulations and in fixed-dose combinations with lamivudine (Combivir) and with abacavir and lamivudine (Trizivir), with no differences in bioavailability or side effects compared to each of the drugs taken separately. The oral forms of zidovudine are rapidly absorbed and undergo extensive first-pass metabolism. The resulting oral bioavailability is ∽64%, and food has no significant impact on oral absorption. Zidovudine reaches a plasma C_{max} at 0.5 to 1.5 h after a 200-mg dose. The mean $t_{1/2}$ of zidovudine in serum is approximately 1 h after oral dosing, but the intracellular $t_{1/2}$ of zidovudine 5′-triphosphate is approximately 3 hours.

Zidovudine has limited plasma protein binding (30–38%). Cerebrospinal fluid (CSF)-to-plasma ratios vary widely (15 to 135%) but average 50% 2 to 4 hours after dosing. The median CSF drug concentrations in adults is 74 μg/l and independent of dose over a dose range of 200 to 1,250 mg. Drug appearance in semen correlates with clearance/suppression of infectious HIV-1 from that compartment. The semen/blood ratios of the concentrations of zidovudine, amprenavir, and lamivudine average 12.9, <1, and 5.3, respectively (86). Zidovudine levels in breast milk and serum are comparable after a single dose. Zidovudine rapidly crosses the placenta by passive diffusion; fetal and maternal concentrations are proportional. Pregnancy does not appear to alter the pharmacokinetics of zidovudine.

Zidovudine is predominantly metabolized hepatically by a uridine diphosphoglucuronosyl transferase (UGT) to its major active metabolite, zidovudine glucuronide, which undergoes renal elimination with a plasma $t_{1/2}$ of 0.61 to 1.73 hours. Urinary recoveries of zidovudine and the glucuronide are 14 and 74%, respectively. Patients with hepatic dysfunction have 2- to 3-fold increases in the peak plasma zidovudine level and elimination $t_{1/2}$ (87). The metabolism of zidovudine also leads to the formation of a minor but cytotoxic compound, 3′-amino-3′-deoxythymidine, which may be partially responsible for the hematologic adverse effects of zidovudine (87). This metabolite has a plasma $t_{1/2}$ of approximately 2.7 h. Renal dysfunction (mean CrCl, 18 ml/min) doubles zidovudine's AUC; total reduced daily dosages of 300 to 400 mg are therefore recommended for

patients with severe renal impairment (CrCl < 15 ml/min). Although zidovudine glucuronide is effectively removed by dialysis, negligible amounts of zidovudine are cleared by either hemodialysis or peritoneal dialysis, and a dose adjustment is recommended in these patients.

Adverse Effects

The most prominent zidovudine toxicities are neutropenia and anemia, which occur at higher doses in 16% and 24% of patients, respectively. In zidovudine-induced anemia, the reticulocyte count is usually depressed and the erythropoietin level is elevated, suggesting inhibition of erythroid stem cell lines. Myelosuppression has been associated with increased doses and duration of zidovudine exposure, as well as with lower baseline hematologic parameters (e.g., CD4 cell count, neutrophil count, hemoglobin concentration, and vitamin B_{12} levels).

Anemia occurs as early as 2 to 4 weeks after initiation of therapy and in nearly 7% of patients with advanced HIV disease, compared to 1% in those with early asymptomatic infection. Although macrocytosis (elevated RBC mean cell volume) occurs in more than 90% of zidovudine recipients, it does not correlate with the development of anemia. Neutropenia is also seen more frequently in advanced HIV infection (37%) than in the early stages (8%) and is usually detected within 6 to 8 weeks after initiation of therapy. In addition to zidovudine dose reduction or discontinuation, management options for zidovudine-related hematologic toxicity include the use of hematopoietic growth factors (erythropoietin or granulocyte colony-stimulating factors).

Zidovudine is also associated with both skeletal and cardiac muscle toxicities. Zidovudine-related polymyositis occurs in approximately 6 to 18% of patients who have been treated for more than 6 months. Clinically, this myopathy is manifested by the insidious onset of myalgias, muscle tenderness, and proximal-muscle weakness, mainly in the lower extremities. Diagnostic features include elevated creatine phosphokinase levels and a myopathic pattern on electromyography. Muscle biopsy reveals minimal to moderate inflammation and myonecrosis with an excess of abnormal mitochondria and a decrease in mitochondrial DNA, probably secondary to the inhibition of DNA polymerase γ. The cessation of zidovudine treatment usually results in a gradual resolution of these abnormalities over the ensuing 6 to 8 weeks.

Nausea, abdominal discomfort, headache, insomnia, malaise, and fatigue are relatively common side effects. These are early symptoms in a substantial number of patients, but often resolve despite continued drug administration. Gastrointestinal effects such as bloating, dyspepsia, hepatitis, and esophageal ulceration have also been described. Zidovudine can cause nail and skin hyperpigmentation. Zidovudine-related seizures and macular edema have been reported as have sometimes fatal cases of lactic acidosis characterized by elevated liver transaminase levels and hepatomegaly due to steatosis.

Zidovudine has been associated with abnormalities of body fat distribution and lipid metabolism (88). In a key study comparing zidovudine/lamivudine with tenofovir/emtricitabine, each with efavirenz in treatment-naïve patients, more patients taking zidovudine experienced loss of limb fat as assessed by dual-energy X-ray absorptiometry scans than in the tenofovir arm. No teratogenicity in human studies has been reported thus far; zidovudine is mutagenic and carcinogenic in rodents.

Drug Interactions

Agents that interfere with the hepatic metabolism or renal excretion of zidovudine can accentuate zidovudine-associated toxicities. Probenecid increases zidovudine levels by inhibiting glucuronidation and renal excretion. Valproic acid, atovaquone, fluconazole, and methadone have been associated with increased zidovudine levels while rifampin and phenytoin can lower zidovudine concentrations. Other drugs which also have myelosuppressive effects, such as ganciclovir, dapsone, flucytosine, and oncological chemotherapeutic agents, may add to the hematologic adverse effects of zidovudine.

Resistance

There are three pathways to zidovudine resistance that can affect multiple NRTIs. In the first, thymidine analog mutations (TAMs) evolve in one of two distinct pathways: (i) T215Y alone or with M41L, L210W (TAM1) and (ii) T215F, which commonly occurs with D67N, K70R, and K219Q or E (TAM2) (89, 90). The level of zidovudine resistance increases with the accumulation of mutations in reverse transcriptase, and susceptibility to most other NRTIs is affected. Two additional multi-nucleoside resistance mutational pathways have been described that confer resistance to multiple NRTIs. The 151 complex affects all NRTIs except tenofovir-based NRTIs: tenofovir disoproxil fumarate and tenofovir alafenamide fumarate. The 151 complex includes A62V, V75I, F77L, F116Y, and Q151M, with the Q151M mutation being the critical mutation for multidrug resistance. The second pathway is the 69 insertion complex, a 2-amino-acid insertion at reverse transcriptase position 69, along with one or more TAMs: M41L, A62V, K70R, L201W, T215 Y or F, and K219 Q or E (28, 91). This mutational complex confers resistance to all current NRTIs. Other amino acid changes at codon 69, without the insertion, can also be associated with resistance to multiple NRTIs.

Two mechanisms of resistance to zidovudine illustrate the complexity of resistance mutational interactions: (i) impairment of the incorporation of the analog into DNA; and (ii) removal of the analog from the prematurely terminated DNA chain (92, 93). Thymidine analog mutations at positions 41, 67, 210, 215, and 219 promote ATP-mediated pyrophosphorolysis with excision of the incorporated terminator (94). The M184V mutation, most associated with lamivudine and emtricitabine resistance, severely compromises pyrophosphorolysis and can result in a resensitization of the virus to zidovudine and other NRTIs, including tenofovir, affected by this pathway of resistance (95).

Most resistance assays evaluate the polymerase domain and do not assess resistance mutations in the connection and RNase H domains of the HIV-1 reverse transcriptase. RNase H cleaves the RNA template as the DNA reverse transcript elongates. Mutations in the RNase H domain increase NRTI resistance, probably by decreasing RNase H activity, allowing more time for excision of zidovudine (96). The clinical relevance of these mutations remains unclear and is complicated by subtype-specific variations in mutations selected in treatment-experienced patients (97). Some mutations, particularly in combination with M184V or other TAMs, can confer reduced susceptibility to zidovudine and nevirapine *in vitro* (98), but thus far they have not been shown to impact response to antiretroviral therapy (99).

Clinical Applications

Zidovudine was the first approved antiretroviral agent and for two decades was one of the agents most widely studied in

clinical trials and used in clinical practice. Zidovudine in combination with another nucleoside analog and either a protease inhibitor (with or without ritonavir enhancement) or a NNRTI became one of the standard-of-care regimens in the era of potent antiretroviral therapy beginning in 1996. As part of initial therapy, the combination of zidovudine and lamivudine was a key dual-NRTI component in multiple clinical trials and demonstrated considerable durability, tolerability, and clinical benefit (100–102).

Comparison of zidovudine with abacavir, in combination with lamivudine and efavirenz, showed similar virologic response in both arms; however, the CD4 cell count rise was greater in the abacavir/lamivudine-treated patients than in the zidovudine/lamivudine-treated patients (103). A comparison of zidovudine/lamivudine with tenofovir/emtricitabine as part of an efavirenz-based regimen in treatment-naïve patients demonstrated lower rates of virologic suppression to <50 copies/ml and higher rates of anemia with the zidovudine regimen (104). Zidovudine/lamivudine has been supplanted by tenofovir/emtricitabine and abacavir/lamivudine as recommended dual-nucleoside components of initial combination regimens for treatment-naïve patients (105).

Zidovudine was the first antiretroviral agent evaluated as a prophylactic agent in HIV-infected pregnant women and accidentally exposed health care workers. In a landmark study (ACTG 076), zidovudine reduced the risk of mother-to-child transmission of HIV-1 by two-thirds compared to placebo (106). For accidentally exposed health care workers, a retrospective case control study found that zidovudine reduced the hazard of HIV transmission by 81% (107). Combination therapy is now, of course, recommended when HIV-1 prophylaxis is indicated.

Lamivudine

Lamivudine [2',3'-dideoxy-3'-thiacytidine; Epivir (Figs. 1 and 3; Tables 1 and 3)] is the (–) enantiomer of a cytidine analog with a sulfur substituted for the 3′ carbon atom in the furanose ring. To streamline the dosing of the nucleoside analog component of combination therapy, fixed-dose formulations of lamivudine/zidovudine (Combivir), lamivudine/zidovudine/abacavir (Trizivir), lamivudine/abacavir (Epzicom), and lamivudine/abacavir/dolutegravir (Triumeq) in single tablets have been approved. The standard dosage of lamivudine is 150 mg twice daily or 300 mg once daily.

Lamivudine displays activity against HIV-1 and -2, as well as hepatitis B virus (HBV). Inhibitory concentrations range from 0.46 ng/ml to 3.45 μg/ml in *in vitro* susceptibility assays. Synergy has been noted in combination with the thymidine analogs and tenofovir.

Mechanism of Action

Although both lamivudine enantiomers have *in vitro* antiviral activity, the (–) enantiomer form is both more potent, possibly on the basis of its resistance to 3′–5′ exonuclease excision, and less cytotoxic because (–) enantiomers of nucleosides are poorly recognized by mammalian polymerases. Lamivudine requires phosphorylation to the triphosphate metabolite for antiviral activity; the triphosphate competitively inhibits the viral reverse transcriptase and interrupts proviral DNA chain elongation.

Pharmacology

Oral lamivudine is rapidly absorbed, and bioavailability in adults is approximately 86%. Peak plasma concentrations range from 2.6 to 5.8 μg/ml after a 300-mg dose. Although absorption of lamivudine is slowed in the postprandial state, there is no significant decrease in the AUC with food. Lamivudine has low protein binding (<36%). The mean plasma elimination $t_{1/2}$ is approximately 2 to 4 hours, but the intracellular $t_{1/2}$ of lamivudine triphosphate varies between 10.5 and 15.5 hours. As a consequence of predominant renal elimination of lamivudine via glomerular filtration and active tubular excretion, dose adjustments are required in individuals with renal impairment.

Adverse Effects

Lamivudine has a very favorable toxicity profile and is well tolerated at dosages ranging from 0.5 to 20 mg/kg/day (108). A trend toward neutropenia is seen only with the highest doses. Insomnia, headache, diarrhea, abdominal pain, and pruritus have been reported but are infrequent (108). Reports of rash, arthralgias, myalgias, pancreatitis, hepatitis, and peripheral neuropathy have an unclear association with lamivudine in adults. In children, rare associations of pancreatitis or hepatitis with lamivudine have been noted.

Drug Interactions

Trimethoprim-sulfamethoxazole decreases the renal clearance of lamivudine and consequently increases its systemic exposure (109). Lamivudine and emtricitabine have nearly identical resistance profiles, have minimal additive antiviral activity, and should not be used together.

Resistance

Resistance to lamivudine develops rapidly and uniformly in treated patients who are not on a fully suppressive antiretroviral regimen or who are poorly adherent through mutations at two codons: 65 and 184. K65R/E/N conveys resistance to lamivudine, as well as several other NRTIs. Mutations at codon 184 that lead to either an isoleucine or a valine substitution for methionine occur rapidly and confer 100- to 1,000-fold decreases in susceptibility (110). The M184V mutation generates lamivudine resistance by decreasing the efficiency of incorporation of lamivudine monophosphate 20- to 100-fold relative to those of the wild-type reverse transcriptase HIV strains (95).

There are potentially beneficial impacts of the M184V mutation. It reverses reduced susceptibility to the thymidine analogs and tenofovir conferred by the TAMs. The M184V mutation, although decreasing the rate of incorporation of lamivudine monophosphate and conferring lamivudine resistance, also reduces pyrophosphorolytic rescue of viral DNA synthesis from chain-terminating zidovudine triphosphate (95). The presence of M184V is also associated with a reduction in viral fitness (111). These effects may, in part, underlie the efficacy of combination therapy of lamivudine or emtricitabine with thymidine analogs and tenofovir.

Although low-level cross-resistance (2- to 4-fold) has been noted with M184V for didanosine and abacavir, the presence of the M184V mutation in isolation does not appear to compromise treatment with didanosine- or abacavir-containing regimens (112, 113). The multidrug-resistance mutations and mutation clusters that impact most NRTIs (69 insertion complex, 151 complex, and TAMs) may also confer various degrees of cross-resistance with lamivudine.

Clinical Applications

Lamivudine (or emtricitabine; see below) is a key component of current initial regimens in treatment-naïve persons

with drug-susceptible virus because of its potency and excellent tolerability. It is typically combined with abacavir or tenofovir (in the case of emtricitabine) to form the dual-nucleoside component of recommended treatment regimens. In patients with viruses containing M184V, there may be a benefit in continuing lamivudine to maintain this mutation because viruses with M184V replicate less well than wild-type viruses when a fully suppressive new regimen cannot be constructed (114). Lamivudine and emtricitabine are essentially clinically interchangeable; the choice depends on which nucleoside or nucleotide analog it is paired with, given the availability of fixed-dose combinations.

Abacavir

Abacavir sulfate {[(1S-cis)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]-2-cyclopent-1-methanol; Ziagen} is a carbocyclic guanine analog (Figs. 1 and 3; Tables 1 and 3). It is available as both a 300 mg tablet and a 20 mg/ml solution, as well as in fixed-dose formulations as lamivudine/abacavir (Epzicom), zidovudine/lamivudine/abacavir (Trizivir), and lamivudine/abacavir/dolutegravir (Triumeq). Abacavir has an IC_{50} of 0.07 μg/ml in human peripheral blood lymphocytes for HIV-1 strains, being additive with NNRTIs, PIs, and other nucleoside analogs.

Mechanism of Action

Abacavir undergoes intracellular metabolism to its active triphosphate form, which interrupts HIV proviral DNA chain elongation. However, abacavir is also a prodrug that, prior to triphosphorylation, requires modification to carbovir. Thus, abacavir activation is characterized by an initial phosphorylation by adenosine phosphotransferase to a monophosphate form, which is then further processed by a cytosolic 5′-nucleotidase to produce carbovir monophosphate; the latter is subsequently converted to di- and triphosphates of carbovir by cellular kinases.

Pharmacology

Abacavir sulfate is well absorbed, with an oral bioavailability of 83% with no important food effects. Similar pharmacokinetic profiles are seen with the abacavir/zidovudine/lamivudine fixed-dose formulation as well as if the three drugs are given separately. The serum C_{max} of 2.87 and 4.73 μg/ml are attained in less than 2 h after dosages of 300 mg b.i.d. and 600 mg, respectively. As the most lipophilic of the nucleoside analogs, abacavir exhibits good central nervous system (CNS) distribution, with mean levels in CSF that are twice the IC_{50} for wild-type HIV-1 and CSF-to-plasma AUC ratios ranging from 27% to 33% (115). Despite a plasma elimination $t_{1/2}$ of 1.2 h for abacavir, the intracellular $t_{1/2}$ of carbovir triphosphate at an abacavir dosage of 300 mg/day is 20.6 h. Thus, the prolonged intracellular $t_{1/2}$ of the active carbovir triphosphate supports the approved twice or once-daily dosing. Abacavir is metabolized to inactive metabolites by alcohol dehydrogenase and glucuronyl transferase with subsequent elimination of metabolites in the urine. Based on increased abacavir AUC and prolonged $t_{1/2}$ in patients with mild hepatic impairment, dosage adjustment is recommended but controversial. Abacavir use in severe hepatic impairment is not recommended.

Adverse Effects

Because abacavir is a relatively selective inhibitor of the HIV reverse transcriptase as compared to cellular DNA polymerases, myelotoxicity is infrequent (116). Abacavir is generally well tolerated, with asthenia, abdominal pain, headache, diarrhea, and dyspepsia being the most commonly reported side effects. Laboratory abnormalities include elevations in liver enzyme levels.

The most significant adverse effect associated with abacavir is the idiosyncratic hypersensitivity reaction. This syndrome has an incidence of approximately 8% and is characterized by the appearance over several days of fever (80%), rash (70%), gastrointestinal symptoms (50%), malaise (40%), and respiratory symptoms (30%). Fever or rash occurs in 98% of cases. Hypersensitivity cases typically occur within 6 weeks of the initiation of abacavir therapy (94%), with a median onset of 11 days. Accompanying laboratory abnormalities can include lymphopenia, thrombocytopenia, and elevated transaminase or creatine phosphokinase levels. This syndrome usually improves within days after the discontinuation of abacavir therapy. Rechallenge in individuals suspected of having this condition is contraindicated because severe allergic reactions, including fatalities, may occur within hours. Individuals who have tolerated abacavir in the past are unlikely to experience hypersensitivity reactions after reinitiating treatment; several severe or fatal exceptions, however, have been reported among these individuals (117, 118). HLA B*5701 predicts abacavir hypersensitivity (119), and screening for HLA B*5701 before initiating the drug is recommended (120, 121). It is important to note, however, that hypersensitivity can still occur in HLA B*5701-negative persons (122). The mechanism of this delayed hypersensitivity appears to be related to abacavir binding to the peptide-binding groove of HLA-B*5701 and presentation of altered class I MHC binding peptides which trigger CD8 T-cell responses (123).

Abacavir use may be associated with an increase in cardiovascular disease and events (124–126). An international cohort study including >23,000 HIV-infected patients reported abacavir as one of the agents associated with increased risk of myocardial infarction (124). A follow-up analysis adjusting for 10-year risk of coronary heart disease also found that abacavir (as well as didanosine) use in the previous 6 months was associated with an increased risk of myocardial infarction. Other studies, however, have failed to identify an association (127, 128), and, as such, the significance of these findings remains controversial. Practically, the potential for such risk should be taken into account when considering treatment of individuals with either a history of cardiovascular disease or a high risk for cardiovascular events.

Drug Interactions

Abacavir and ethanol share a similar metabolic pathway, and coadministration has been shown to increase the abacavir AUC by 41% in males. A small study examining the interaction of abacavir with methadone suggested that methadone clearance increased by 22%, which may only be significant in a very small proportion of patients.

Resistance

Selection for resistance to abacavir *in vitro* is characterized by the accumulation of multiple mutations in the HIV-1 reverse transcriptase. An initial M184V mutation, conferring a 2- to 5-fold increase in resistance, is followed by the appearance of either L74V and K65R/E/N or L74V and Y115F. While individually these mutations are associated with a low-level (2- to 4-fold) loss of susceptibility, M184V-containing double mutants have increased (7- to 11-fold) resistance. Abacavir-resistant isolates containing combinations of these

mutations have various degrees of cross-resistance to lamivudine and didanosine.

Isolates obtained after abacavir monotherapy revealed mutations at codons 65, 74, and 184. The addition of zidovudine delayed the selection of the M184V mutation. *In vivo* studies identified the following mutations: K65R, L74V, Y115F, and M184V, as well as TAMs M41L, D67N, K70R, L210W, T215F/Y, and K219E/Q; the Q151M complex, usually in combination with V75I, F77L, F116Y; and T69 insertion mutations (28). Increasing numbers of TAMs (>4) result in a loss of virologic response to abacavir (129). Multinucleoside analog resistance-associated insertions at position 69 and the 151 complex also confer cross-resistance to abacavir (28).

Clinical Applications

Abacavir has demonstrated durable efficacy in combination with lamivudine and a third active antiretroviral drug (a boosted PI, NNRTI or INSTI) in treatment-naïve patients with baseline HIV-1 RNA levels <100,000 copies/ml (103, 105,130–134). Abacavir may be administered either as a separate pill or in a more convenient fixed-dose combination, single-pill formulation that might favorably impact drug adherence. Abacavir-lamivudine and zidovudine-lamivudine regimens, each combined with efavirenz in treatment-naïve individuals, show comparable rates of virologic suppression to <50 copies/ml, but there is a greater increase in CD4 cell counts in the abacavir-lamivudine group (209 cells/mm^3 versus 155 cells/mm^3) (103, 135). A study that compared abacavir-lamivudine to tenofovir disoproxil-emtricitabine, both regimens combined with efavirenz, demonstrated a lower proportion of patients achieving virologic suppression to <50 copies/ml of HIV-1 RNA but similar adverse event rates in both arms. In combination with lopinavir/ritonavir, abacavir-lamivudine is noninferior to tenofovir disoproxil-emtricitabine (136). Data from ACTG A5202, a study comparing abacavir-lamivudine with tenofovir-emtricitabine in combination with either efavirenz or atazanavir-ritonavir in treatment-naïve patients, showed that individuals with baseline HIV-1 RNA levels ≥100,000 copies/ml were more likely to experience virologic failure in the abacavir-lamivudine arm than in the tenofovir-emtricitabine arm; there was no difference in time to virologic failure for participants with HIV-1 RNA levels <100,000 copies/ml (137, 138). When compared to a single tablet regimen of efavirenz/tenofovir disoproxil/emtricitabine, the single tablet combination of dolutegravir/abacavir/lamivudine led to higher proportions of participants with HIV-1 RNA levels <50 copies/ml at 48 weeks, shorter median time to viral suppression, and fewer adverse events (139). For the treatment of antiretroviral-experienced patients, the use of abacavir-containing regimens has also demonstrated clinical utility (140, 141).

Emtricitabine

Emtricitabine {5-fluoro-1-(2R,5S)-2-[hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine; Emtriva} is a cytidine analog that is the (–) enantiomer of a thio analog of cytidine; it differs from other cytidine analogs in that it has a fluorine in the 5 position (Figs. 1 and 3; Tables 1 and 3). It is available as both a 200 mg capsule and a 10 mg/ml oral solution, as well as in multiple fixed-dose formulations with TDF (Truvada), with TDF and efavirenz (Atripla), with TDF and rilpivirine (Complera), with TDF, elvitegravir, and cobicistat (Stribild), with tenofovir alafenamide (TAF) (Descovy), with TAF and rilpivirine (Odefsey), and with TAF, elvitegravir, and cobicistat (Genvoya). Emtricitabine is administered in a once-daily dosage of 200 mg.

Emtricitabine is 4- to 10-fold more potent than lamivudine; for a laboratory-adapted strain, it has an IC_{50} of 0.002 to 0.124 μg/ml in human cell lines (142). This heightened potency may be the result of a greater efficiency of incorporation of emtricitabine triphosphate than of lamivudine triphosphate by the HIV-1 reverse transcriptase (143). In addition, emtricitabine triphosphate has a longer intracellular $t_{1/2}$ (see below). The drug has activity against HIV-1, HIV-2, and HBV. The role of emtricitabine as an anti-HBV agent is discussed in chapter 13. Emtricitabine has demonstrated synergistic or additive activity with other nucleoside analogs, PIs, and NNRTIs.

Mechanism of Action

Emtricitabine undergoes serial intracellular phosphorylations to an active triphosphate form that inhibits the activity of the HIV-1 reverse transcriptase by competing with the natural substrate deoxycytidine 5′-triphosphate. Because it lacks a hydroxyl group at the 3′ position of the oxothiolane moiety (144), incorporation of emtricitabine 5′-triphosphate interrupts HIV proviral DNA chain elongation in susceptible strains (143).

Pharmacology

Emtricitabine is rapidly absorbed with high oral bioavailability (∽93%) that is not affected by coadministration of food. After a 200-mg dose, the mean peak concentration is 1.8 μg/ml. The oral bioavailability of the oral solution is lower (∽75%) and as such requires a slighter higher daily dose. The drug has low protein binding (<4%). The plasma elimination $t_{1/2}$ averages approximately 10 h, and the intracellular $t_{1/2}$ of emtricitabine triphosphate is longer than 20 h, allowing once daily dosing. Emtricitabine is predominantly renally eliminated by both glomerular filtration and active tubular secretion; thus, dosing interval adjustments are required in individuals with renal impairment.

Adverse Effects

Emtricitabine, like lamivudine, is well tolerated. Adverse effects attributed to emtricitabine have been mild to moderate CNS and gastrointestinal symptoms (Table 3). Skin hyperpigmentation is also possible and is more common (as high as 32%) in children. Exacerbations of hepatitis B in coinfected individuals have been reported after discontinuation of emtricitabine.

Drug Interactions

There are no clinically significant drug interactions.

Resistance

As with lamivudine, high-level resistance to emtricitabine is associated with K65R/E/N and M184I/V reverse transcriptase mutations (28). The combination of tenofovir with emtricitabine in clinical studies was less likely to select for the M184V mutation than the zidovudine-lamivudine combination. The emtricitabine-containing regimen may also be less likely to select for the K65R mutation than lamivudine in the presence of tenofovir (145).

Clinical Applications

Emtricitabine is recommended as a component of initial antiretroviral therapy for treatment-naïve patients and can be used interchangeably with lamivudine but should never

be used in combination with lamivudine (105). Emtricitabine has also shown efficacy in treatment-experienced patients with plasma HIV-1 RNA levels of < 400 copies/ml who switched from lamivudine- or PI-containing regimens to emtricitabine-containing ones. The simplification to once-daily emtricitabine does not affect the proportion of patients with plasma HIV-1 RNA level suppression to < 400 or < 50 copies/ml. Emtricitabine in combination with TDF has demonstrated considerable potency and durability of virologic control as part of PI- or NNRTI-based regimens (146).

SELECTED INVESTIGATIONAL NRTIS

Nucleoside analog drug development continues with the goals of improving safety, pharmacokinetics, potency, and activity against drug-resistant strains. Selected new agents in this drug class are listed in Table 2.

NUCLEOTIDE ANALOG REVERSE TRANSCRIPTASE INHIBITORS

Tenofovir Disoproxil Fumarate (See also Tenofovir Alafenamide)

Tenofovir {9-[(R)-2-[[bis[[(isopropoxycarbonyl)oxy]methoxy]-phosphinyl]methoxy]propyl]adenine fumarate; Viread (Figs. 1 and 3; Tables 1 and 3)} is a prodrug of the cyclic nucleoside phosphonate 9-R-(2-phosphonomethoxy-propyl)adenine (PMPA). This agent is approved for once-daily dosing and is available in 300-mg tablets and powder for oral use. It is available in combination with emtricitabine (Truvada), with emtricitabine and efavirenz (Atripla), with emtricitabine and rilpivirine (Complera), and with emtricitabine and elvitegravir and cobicistat (Stribild) in fixed-dose combinations. Tenofovir disoproxil fumarate (TDF) is an inhibitor of retroviruses, including HIV-1, simian immunodeficiency virus, and feline leukemia virus, as well as hepadnaviruses.

Mechanism of Action

TDF is converted to tenofovir by diester hydrolysis and phosphorylation to form tenofovir diphosphate. As a nucleotide analogue, only two intracellular phosphorylation steps are needed to convert the drug to its active form. The mechanism of reverse transcriptase inhibition is as described for the nucleoside analogs.

Pharmacology

Because of the drug's hydrophilic properties, tenofovir demonstrates a permeability-limited oral absorption and has a bioavailability of 25 to 40% as the *bis*-ester prodrug, TDF (147). The AUCs of the tablet and oral powder formulations are similar despite a lower C_{max} for the oral powder. Administration of TDF with a high-fat meal increases the AUC of tenofovir by ∽40%, an effect not seen with a lighter meal. Tenofovir has a plasma $t_{1/2}$ of approximately 17 hours and an intracellular $t_{1/2}$ of the diphosphate form of 12 to 15 hours in activated PBMCs and 33 to 50 hours in resting PBMCs, thus permitting once-daily dosing. The drug is excreted renally by both glomerular filtration and active tubular secretion and requires dosage adjustment in patients with renal impairment.

Adverse Effects

A small proportion of patients taking TDF experience increases in serum creatinine, glycosuria, hypophosphatemia, and acute tubular necrosis. However, renal impairment develops uncommonly in patients with normal renal function at baseline (148). Patients with more advanced HIV disease, greater treatment experience, concomitant PI use, and preexisting renal impairment may be at an increased risk of this complication (149). The mechanism may be a direct toxic effect of free tenofovir in renal tubular cells (150). The agent should be used with caution in individuals with preexisting renal disease, and alternative nucleoside analogs should be considered. In individuals coinfected with HBV, discontinuation of TDF may cause flares of viral replication and hepatitis; close monitoring of such patients is advised.

Fat maldistribution is not commonly seen with TDF-based regimens (151), and switching from zidovudine- or stavudine-containing regimens to TDF or abacavir may slow or halt the progression of this syndrome. TDF may have a more pronounced effect on bone loss than other agents (152). While low bone mineral density and fractures appear more common in HIV patients compared to non-HIV patients, the impact of antiretrovirals on loss of bone mineral density suggests a contribution of antiretroviral therapy, which may be more noteworthy with agents such as TDF and PIs (153–156). The safety of tenofovir in pregnancy has not been definitively established, and some animal studies have reported impaired fetal growth and decreased fetal bone porosity within 2 months of starting maternal therapy. However, data from infants exposed *in utero* to tenofovir do not suggest an increase in growth or bone abnormalities (157). The drug is now routinely used in pregnancy.

Drug Interactions

TDF decreases the AUC of atazanavir by 25%; therefore, patients who receive concomitant therapy with TDF should only use ritonavir- or cobicistat-boosted atazanavir. The combination of TDF and didanosine has been associated with increased didanosine toxicity and a high rate of early virologic failure as well as CD4 cell decline in treated individuals, although conflicting data exist on this point (158, 159). Coadministration of TDF with ritonavir-boosted PIs may result in increased tenofovir absorption by ∽30% due to inhibition of p-glycoprotein.

Resistance

Tenofovir retains activity against a range of nucleoside-resistant HIV strains. *In vitro*, the K65R/E/N mutation of HIV-1 confers a 9-fold decrease in sensitivity to tenofovir; the M184V mutation partially reverses this resistance. The K70E mutation and insertions at codon 69 also confer decreased susceptibility to tenofovir (160). The effect of the 69 insertion complex is potentiated in the presence of the M184V mutation. Nonresponse to tenofovir is more likely to occur when the drug is added to a failing regimen in heavily pretreated patients, as opposed to its use as a part of a new regimen with other active medications. Clinical data suggest that the presence of > 2 TAMs, including M41L or L210W, is associated with decreased susceptibility to tenofovir. The activity of tenofovir is retained in the presence of the 151 complex (161, 162).

Clinical Applications

TDF has been an essential component of initial treatment regimens for treatment-naïve patients, that traditionally were combined with lamivudine or emtricitabine plus an agent from one of three other classes: NNRTIS, PIs, or INSTIs. Its availability in multiple fixed-dose drug combinations also increases its clinical utility. Potent and durable virologic suppression occurs in treatment-naïve individuals

with TDF and lamivudine or emtricitabine in combination with efavirenz (151). In direct comparison with lamivudine-zidovudine, TDF-emtricitabine demonstrates a greater increase in CD4 cell counts and a higher rate of virologic suppression to <50 copies/ml sustained over 144 weeks (163). As described above, compared to abacavir-lamivudine in combination with either efavirenz or atazanavir-ritonavir, TDF-emtricitabine shows a more favorable virologic efficacy and side-effect profile.

In treatment-experienced individuals with incomplete virologic suppression and baseline nucleoside resistance mutations, the addition of TDF to stable regimens has resulted in significant reductions in plasma HIV-1 RNA levels (164) with the caveat that incremental monotherapy is discouraged. In patients with suppressed viral loads at baseline, switching the nucleoside component to TDF-emtricitabine results in fewer treatment failures and drug discontinuations than switching to abacavir-lamivudine (165).

TDF is also recommended, in combination with emtricitabine in a fixed-dose combination, as preexposure prophylaxis for prevention of HIV infection in high-risk populations including sexually active men who have sex with men, heterosexually active men and women at substantial risk for HIV infection, injection drug users, and heterosexually active men and women whose partners are living with HIV (166, 167).

Tenofovir Alafenamide

Tenofovir alafenamide [isopropyl (2S)-2-[[[(1R)-2-(6-aminopurin-9-yl)-1-methyl-ethoxy]methyl-phenoxy-phosphoryl]amino]propanoate; GS-7340] is a nucleotide reverse transcriptase inhibitor prodrug of tenofovir associated with reduced risk of renal adverse effects. It is currently available only in fixed-dose combinations with emtricitabine (Descovy), with emtricitabine and rilpivirine (Odefsey), and with emtricitabine, elvitegravir, and cobicistat (Genvoya).

Mechanism of Action

Tenofovir alafenamide is 1,000- and 10-fold more active against HIV *in vitro* than tenofovir and TDF, respectively. Conversion to tenofovir occurs intracellularly with higher active metabolite concentrations seen in peripheral blood mononuclear cells (PBMCs) than with TDF. Tenofovir enters cells where it undergoes ester hydrolysis by lysosomal carboxypeptidase cathepsin A (CatA). A key intermediate formed with alanine eventually releases tenofovir, which is subsequently phosphorylated to the active metabolite tenofovir-diphosphate. Intracellular cleavage by CatA occurs rapidly in HIV-target cells, and this, coupled with metabolites that are trapped in cells, results in accumulation of the active metabolite tenofovir diphosphate (168).

Pharmacology

Tenofovir alafenamide is rapidly absorbed, reaching mean peak concentrations of 0.16 μg/ml within 1 hour. Administration with a high fat meal increases the AUC ∽85%. Tenofovir alafenamide is ∽80% bound to plasma proteins. Tenofovir alafenamide is primarily metabolized first by CatA in PBMCs and macrophages and then by carboxylesterase 1 in hepatocytes. The terminal plasma $t_{1/2}$ is ∽30 minutes but that of tenofovir diphosphate is ∽150–180 hours within PBMCs. Renal impairment has no impact on concentrations of TAF. Tenofovir alafenamide results in 90% lower serum tenofovir levels compared to TDF with consequently diminished renal and bone toxicity.

Adverse Effects

The most common adverse effect reported during clinical trials was nausea (≥10%). Of note, TAF has been associated with smaller mean serum creatinine increases, less proteinuria, and a smaller decrease in bone mineral density at the spine and hip than TDF (169, 170).

Drug Interactions

Tenofovir alafenamide is a substrate of P-glycoprotein, and drugs that strongly affect P-glycoprotein activity may alter TAF absorption. Coadministration of tipranavir/ritonavir, carbamazepine, oxcarbazepine, phenobarbital, phenytoin, rifamycins, and St. John's wort with TAF is not recommended.

Resistance

As TAF is a prodrug of tenofovir, the resistance pattern is identical to that of TDF (171). Because TAF achieves higher intracellular concentrations and is more potent than TDF, there is speculation that it may retain activity against viral strains with known mutations, but confirmatory clinical data are lacking at this time (172).

Clinical Applications

Tenofovir alafenamide is included in the regimens recommended for first-line therapy of treatment-naïve patients with estimated creatinine clearances of ≥30 ml/min, in the form of the fixed-dose combination elvitegravir/cobicistat/TAF/emtricitabine (105). The side-effect profile of TAF is favorable compared with TDF when in combination with elvitegravir-cobicistat or darunavir-ritonavir, including improved renal and bone safety parameters (173, 174). It is an increasingly popular clinical choice, and it is likely that the alafenamide form of the prodrug will replace the disoproxil fumarate form.

NONNUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS

Although structurally diverse, the NNRTIs share similar HIV-1 reverse transcriptase binding sites and function by noncompetitive allosteric binding to a hydrophobic pocket created by the p66 palm-and-thumb subdomains of the reverse transcriptase (175). This alters the structure and function of the HIV-1 reverse transcriptase (176). When the NNRTI is bound to this site, it interacts with multiple amino acid residues within the pocket. Mutations of these key amino acids alter inhibitor binding properties and form the mechanistic basis for NNRTI resistance. The binding of an NNRTI to the enzyme-DNA complex is proposed to slow reverse transcriptase-mediated catalysis by causing a distortion of the normal spatial configuration of the carboxyl groups and associated Mg^{2+} ions of three aspartic acid residues in the adjacent polymerase active site (177). As a consequence, nucleotide triphosphate molecules destined to be added to the elongating proviral DNA chain are bound but inefficiently utilized. The narrow spectrum of antiviral activity is conferred by a tyrosine at amino acid position 181 in HIV-1, but which is not present in HIV-2 or the HIV-1 O clade (subtype), against which these compounds are inactive (178).

Nevirapine

Nevirapine (11-Cyclopropyl-4-methyl-5,11-dihydro-6H-dipyrido[3,2-b:2',3'-e][1,4]diazepin-6-one; Viramune) (Figs. 1

and 4; Tables 1 and 3) formulations include 200-mg tablets, a once-daily extended release 400 mg tablet, and a 50 mg/5 ml oral suspension. Nevirapine antiviral activity is limited to HIV-1, with an IC_{50} of 0.003 μg/ml to 0.03 μg/ml, while 50% cytotoxicity concentrations are more than 300-fold higher.

Pharmacology

Nevirapine is rapidly absorbed with an oral bioavailability of >90%. An initial serum C_{max} of 2.0 μg/ml (7.5 μM) is reached approximately 4 hours after a 200-mg oral dose. Steady-state trough concentrations average 4.5 μg/ml (17 μM) at a nevirapine dosage of 400 mg/day. Concentrations in plasma do not appear to be altered by food or antacids. Secondary peak levels are seen, which may reflect enterohepatic recirculation. Plasma protein binding is approximately 60%. Nevirapine is highly lipophilic, and CSF concentrations are about 45% of plasma values (179). Nevirapine crosses the placenta and is present in breast milk and semen.

Nevirapine is metabolized by CYP3A and CYP2B6 and also induces these enzymes. The clearance of nevirapine increases through the first 2 to 4 weeks of dosing, such that its plasma elimination $t_{1/2}$ decreases from 45 hours after single doses to 25 to 30 hours with multiple dosing. Detectable levels of nevirapine have been found in chronically treated children and adults for as many as 7 days after cessation (180). Detectable levels may persist longer in treatment-naïve women who receive single-dose nevirapine for prophylaxis of mother-to-child transmission of HIV-1.

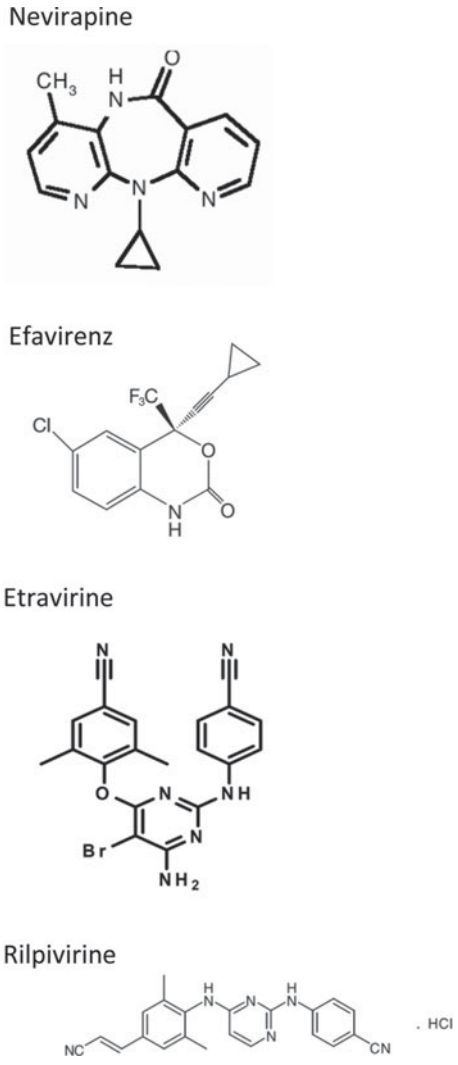

FIGURE 4. Chemical structures of HIV nonnucleoside reverse transcriptase inhibitors.

Adverse Effects

The most common adverse reaction associated with nevirapine is a nonpruritic macular rash distributed on the face, trunk, and extremities, excluding the palms and soles; the rash usually occurs within the first 6 weeks of therapy. Rash has occurred in 48% of nevirapine recipients who received 400 mg/day. The rash may be accompanied by hepatotoxicity, fever, and myalgias.

The incidence of rash does not appear to be related to plasma drug levels, stage of HIV disease, history of rash with other agents, sex, or race. The risk may be attenuated by administering a lower initial dosage of 200 mg/day for 2 weeks and then increasing the dosage to 200 mg b.i.d. Liver toxicity occurs in about 4% of patients. The risk of hepatotoxicity is higher in women and in patients with higher CD4 cell counts (>250 cells/mm^3 in women and >400 cells/mm^3 in men), and thus nevirapine should not be initiated in these populations. Coinfection with HBV or HCV increases the risk of liver toxicity. Life-threatening and fatal cases of hepatotoxicity, as well as severe, even fatal, skin reactions, including Stevens-Johnson syndrome, toxic epidermal necrolysis, and hypersensitivity reactions, have been reported and can occur during the first 18 weeks of therapy. The overall rate of hypersensitivity reactions has been about 6%; increased risk has been associated with high pretreatment plasma HIV-1 RNA levels, high CD4 cell counts, and female sex.

Drug Interactions

Nevirapine induces the hepatic CYP450 enzyme. As such, it affects its own metabolism as well as coadministered drugs that also utilize the CYP3A4 metabolic pathway. Nevirapine decreases the AUCs of the PIs. Coadministration of rifampin and nevirapine is associated with lower nevirapine concentrations and greater variability in plasma drug levels. Some data suggest that combining efavirenz or nevirapine at standard doses in combination with rifampin results in comparable virologic and clinical outcomes (181, 182). Nevirapine-induced methadone withdrawal has been reported, and the methadone dose should be increased before commencing nevirapine (183).

Resistance

The nevirapine binding site is created mainly by amino acid residues at positions 100 to 110 and 180 to 190, comprising the 2β sheets that form the binding pocket in the HIV-1 reverse transcriptase. Consequently, mutations associated with resistance generally fall within these regions. *In vitro* and clinical isolate data indicate mutations conferring nevirapine resistance include L100I, K101P, K103N/S, V106A/M, V108I, Y181C/I, Y188C/L/H, G190A, and M230L (28). Several of these engender variable cross-resistance to other NNRTIs (184).

In vivo, nevirapine-resistant isolates are genetically mixed populations, and the most common ones in patients with virologic failure occur at positions 103, 181, 190, 188, and 106. Each mutation reduces nevirapine susceptibility at least 50-fold (28). The nevirapine-associated Y181C substitution

in zidovudine-resistant HIV strains increases zidovudine susceptibility *in vitro*. Mutations at positions 138 and 227, selected by etravirine, appear to reduce susceptibility to nevirapine and efavirenz, and V179F in combination with Y181C causes high-level resistance to nevirapine and etravirine (185).

Clinical Applications

Nevirapine's role as part of initial antiretroviral regimens continues to diminish, but its historic position as the lead compound in the NNRTI class is well established. In a direct comparison with efavirenz and nevirapine, both given in combination with stavudine and lamivudine, nevirapine demonstrated a similar rate of virologic suppression to a plasma HIV-1 RNA level of < 50 copies/ml, without differences in treatment failure between regimens (186). Despite these data, nevirapine's twice-daily dosing and risk of heptatotoxicity make it a less attractive option as initial therapy than efavirenz or other NNRTIs. The prevalence of NNRTI-resistant virus in treatment-naïve patients also limits nevirapine's utility as first-line treatment (105). In low- and middle-income countries, nevirapine still plays a role in initial regimens because of its low cost and availability in fixed-dose generic combinations (187).

Nevirapine is safe in women with CD4 cell counts < 250 cells/mm^3, including pregnant women. Administering single-dose nevirapine to both the mother and infant in the peripartum period significantly reduces vertical transmission of HIV-1 but is associated with a substantial incidence of nevirapine-resistance emergence in women (up to 40 to 60%) and infants (33 to 87%), especially if techniques for low-frequency viral variant detection are employed (188, 189). Addition of a short course of nucleoside analogs (zidovudine-lamivudine or tenofovir-emtricitabine) decreases the development of maternal and pediatric resistance after single-dose nevirapine (190, 191). For infants infected by mother-to-child transmission after exposure to nevirapine, a strategy of induction and virologic suppression with a lopinavir/ritonavir-based regimen, followed by transition to nevirapine-based treatment, is associated with long-term virologic response, sustainable up to three years, and is not significantly impacted by resistance (192, 193). Advances in access and guideline advice have made these approaches outmoded, but the principles are important to remember when women present at term or in labor and a new HIV diagnosis is made in that setting.

Efavirenz

Efavirenz [(4S)-6-chloro-4-(2-cyclopropylethynyl)-4-(trifluoromethyl)-1H-3,1-benzoxazin-2-one; L-743,726, DMP 266, Sustiva] is available in tablet and capsule formulations (Figs. 1 and 4; Tables 1 and 3). It is also available as a fixed-dose combination tablet with emtricitabine and tenofovir (Atripla). It has an IC_{90} ranging from 0.95 μg/l to 2.84 μg/l for wild-type clinical and laboratory HIV-1 isolates.

Pharmacology

The bioavailability of efavirenz in humans has not been reported. Administration with food increases the efavirenz AUC by ∽17–28% while increasing the plasma C_{max} more significantly, as high as 79%. The plasma C_{max} at steady-state averages 1.26 mg/l and the C_{min} averages 0.5 mg/l at a 600-mg daily dosage. Efavirenz is highly protein bound (> 99%), and CSF concentrations are only about 0.26 to 1.19% of plasma concentrations. Efavirenz is primarily metabolized by CYP3A and CYP2B6 to hydroxylated metabolites, with subsequent glucuronidation, and these metabolites are excreted in the urine. The terminal $t_{1/2}$ is approximately 40–55 hours after multiple doses. Efavirenz achieves therapeutic concentrations in semen throughout the dosing interval and can suppress HIV-1 levels in the seminal compartment.

Adverse Effects

Efavirenz toxicities include maculopapular rash, dizziness (27% versus 9% in the control group), impaired concentration (11% versus 4%), depression (9% versus 5%), abnormal dreaming (6% versus 1%), and euphoria (5% versus 2%). CNS adverse events occur more often in subjects taking efavirenz (54% versus 27%), although these symptoms tend to resolve over the course of a few weeks, probably due to self-induction of hepatic metabolism and increased clearance of the drug. The incidence of adverse events does not differ with the timing of the dose, splitting of the dose, or age of the subject, although when efavirenz is taken at night, the CNS side effects may be more tolerable. Controversy remains whether efavirenz increases the propensity for suicide, but more recent data have failed to find such an association (194–196). The incidence and severity of rash are not related to dose. Efavirenz administration can generally be continued despite the occurrence of a rash, which resolves over 3 to 4 weeks. As with other NNRTIs, metabolic abnormalities, such as lipoatrophy and elevations in high-density lipoprotein cholesterol and total cholesterol, occur in subjects taking efavirenz (103, 197). Efavirenz has been associated with an increased risk of lipoatrophy compared to lopinavir-ritonavir, whereas derangements in lipid profiles (triglycerides in particular) were similar in the two groups (197).

Efavirenz has been reported to cause neural tube defects in infants whose mothers have been exposed to efavirenz during the first trimester, although a causal relationship is not established (198). In pregnant cynomolgus monkeys treated throughout pregnancy with doses of efavirenz resulting in plasma levels similar to those in humans dosed with 600 mg/day, craniofacial malformations (anencephaly, unilateral anopthalmia, microphthalmia, and cleft palate) occur. Recommendations for the use of efavirenz in pregnancy have evolved since the drug was first approved. Women who wish to become pregnant or who are in their first trimester should not be started on efavirenz if alternatives are readily available. However, for women who are already on efavirenz and have been pregnant for 6–8 weeks by estimated dates, it is reasonable to continue the efavirenz as the risk period for neural tube defects will have passed (199, 200). The global rollout of antiretroviral therapy has provided reassurance concerning the potential teratogenic effects of efavirenz as clusters of cases of birth defects have not been reported. The recent prevalence of birth defects following first trimester efavirenz exposure is 2% with a relative risk of birth defects in efavirenz-containing regimens to non-efavirenz-based regimens of 0.85 (95% CI 0.61–1.20) (201). Women should undergo pregnancy testing before starting efavirenz and should receive counseling regarding contraceptive practices.

Drug Interactions

Efavirenz is a substrate of CYP450 and thus affects the hepatic metabolism of many coadministered drugs. It has been characterized as an inducer of CYP3A and CYP2B6 and an

inhibitor of CYP2C9 and CYP2C19. When coadministered with protease inhibitors, efavirenz may decrease protease inhibitor AUCs and boosting is recommended. Efavirenz also significantly decreases AUCs of maraviroc, simeprevir, azole antifungals, statins, oral contraceptives, and methadone. Coadministration with rifabutin requires a 50% dose increase of rifabutin, while coadministration with rifampin requires a dose increase of efavirenz to 800 mg once daily in patients >50 kg based on pharmacokinetic modeling data.

Resistance

Efavirenz selects for multiple reverse transcriptase mutations: L100I, K101P, K103N/S, V106M, V108I, Y181C/I, Y188L, G190S/A, P225H, and M230L (28). The K103N mutation confers cross-resistance to nevirapine but not to etravirine or rilpivirine. Isolates containing the K103N or G190S mutations from patients failing efavirenz combination therapy have shown 19- to 36-fold or 280-fold reduced susceptibility *in vitro*, respectively. Resistance to efavirenz increases to 100-fold with the development of additional mutations at codons 106, 188, and 190 (202). Several nucleoside analog mutations (e.g., 118I, 298Y, and 215Y) can result in hypersusceptibility to efavirenz in patients with extensive NRTI experience (203). The underlying mechanism may include increased reverse transcriptase enzyme susceptibility to the NNRTI (e.g., with 118I/215Y) or decreased virion-associated levels of reverse transcriptase (with 208Y/215Y and 118I/208Y/215Y).

Clinical Applications

Current clinical practice has moved away from the use of efavirenz in antiretroviral-naïve patients initiating treatment because of its CNS toxicities (105) and the availability of integrase strand-transfer inhibitors. However, the virologic efficacy of efavirenz-based regimens at 24 weeks remains comparable to many other recommended regimens. Efavirenz-containing regimens compare favorably to PI-based therapy, including atazanavir and lopinavir/ritonavir-based regimens (100, 101, 204). In combination with the dual-nucleoside backbone of tenofovir-emtricitabine, zidovudine-lamivudine, or abacavir-lamivudine, efavirenz has achieved high rates of virologic suppression (103, 151, 205), and the fixed-dose combination of tenofovir/emtricitabine/efavirenz demonstrated superior virologic suppression and CD4 cell-count response when compared with the combination of zidovudine-lamivudine plus efavirenz (206). Efavirenz compared with nevirapine, each in combination with lamivudine-stavudine, showed no significant differences in rates of virologic suppression or increases in CD4 cell counts (186). Efavirenz-based regimens showed comparable rates of virologic failure to atazanavir-ritonavir-based regimens in the ACTG 5202 study (137) but were less likely to be associated with virologic failure than lopinavir/ritonavir based regimens in the ACTG 5142 study (207). Efavirenz in its single-tablet regimen formulation (tenofovir/emtricitabine/efavirenz) was noninferior to the elvitegravir/cobicistat/tenofovir/emtricitabine fixed-dose regimen (208). Comparisons to rilpivirine are discussed in that section.

Etravirine

Etravirine [4-({6-amino-5-bromo-2-[(4-cyanoanilino)pyrimidin-4-yl}oxy)-3,5-dimethylbenzonitrile; Intelence, TMC 125] (Figs. 1 and 4; Tables 1 and 3) is a diaryl-pyrimidine derivative that binds to HIV reverse transcriptase with mutations within the hydrophobic pocket. It is approved for use in individuals who have experienced virologic failure with other NNRTIs and harbor a multidrug-resistant virus (209). Formulations of this agent include 25-mg, 100-mg, and 200-mg tablets. Etravirine exhibits activity against laboratory as well as wild-type strains of HIV-1, with a median IC_{50} ranging from 0.9 to 5.5 nM (0.4 to 2.4 ng/ml).

Pharmacology

The oral bioavailability of etravirine has not been reported. Its plasma AUC is diminished by 50% under fasting conditions; therefore, etravirine should be taken with food. The plasma C_{max} averages 390 ng/ml at 4 hours after dosing. It is highly protein bound, and its distribution in other compartments (CSF and genital tract secretions) has not been evaluated to date. Etravirine is metabolized by the CYP3A, CYP2C9, and CYP2C19 enzymes. Although the plasma elimination $t_{1/2}$ of etravirine (41 hours) is long enough to support once-daily dosing, such dosing has not been tested in controlled trials to date. It is primarily excreted via feces, with only a limited amount of renal excretion (1.2%).

Adverse Effects

The most commonly reported adverse effects are nausea and rash (210). The rash is mild to moderate in severity, occurs most frequently in the second week of therapy, and generally resolves within 1 to 2 weeks on continued therapy. It is a rare cause of drug discontinuation (210–212). There are no observable effects on lipids or liver function enzymes.

Rare cases of serious skin reactions, including Stevens-Johnson syndrome and erythema multiforme, were reported during clinical development, but the risk appears to be low (no cases among over 500 treated patients in phase III studies). Patients with a history of NNRTI-related rash did not appear to be at an increased risk for the development of rash while taking etravirine.

Drug Interactions

Like nevirapine and efavirenz, etravirine is metabolized by the CYP450 system. It is both a substrate and an inducer of CYP3A4, as well as an inhibitor of CYP2C9, CYP2C19, and p-glycoprotein. As a result, coadministration of etravirine with inhibitors or inducers of these CYP450 enzymes may alter levels of etravirine in serum. Conversely, coadministration of etravirine with drugs that are substrates of these enzymes may alter their levels in serum. Certain antiretrovirals should not be coadministered with etravirine, including other NNRTIs, all unboosted PIs, and ritonavir-boosted tipranavir or fosamprenavir. Dolutegravir concentrations are significantly decreased by etravirine, so dolutegravir should only be administered with etravirine when coadministered with atazanavir-ritonavir, darunavir-ritonavir, or lopinavir/ritonavir. The AUC of maraviroc is decreased by 53%, requiring dosage adjustment to 600 mg twice daily if coadministered with etravirine. If maraviroc is dosed with a ritonavir-boosted PI as well as etravirine, the maraviroc dosage is 150 mg twice daily. Etravirine can be combined with the integrase inhibitor raltegravir without dose adjustments.

Resistance

Multiple reverse transcriptase mutations present pretreatment affect the response to etravirine: V90I, A98G, L100I, K101E/H/P, V106I, E138A/G/K/Q, V179D/F/T, Y181C/I/V, G190A/S, and M230L (28, 210,213–215). Most data on

etravirine resistance are derived from studies of treatment-experienced patients where it is used in combination with darunavir-ritonavir. Algorithms have been developed that predict etravirine susceptibility by assigning a score based on genotypic and phenotypic cutoffs (216). The L100I, K101P, or Y181C/I/V are considered "major" mutations as the presence of any of these alone confers decreased susceptibility to etravirine *in vitro*, and is associated with decreased virologic response at 24 weeks for treatment-experienced patients receiving an etravirine-based regimen (217). The presence of at least three of these mutations at baseline (particularly V179D/F, Y181V, and G190S) has been associated with a diminished drug response (211, 212). The highest levels of resistance to etravirine *in vitro* were observed for HIV-1 harboring a combination of mutations: V179F plus Y181C (187-fold change), V179F plus Y181I (123-fold change), or V179F plus Y181C plus F227C (888-fold change). The K103N mutation does not confer resistance to etravirine. Cross-resistance within the NNRTI class is common. Patients may develop resistance to etravirine if failing nevirapine or efavirenz-based regimens (218), and the development of resistance to etravirine was seen in 90% of patients failing a rilpivirine-based regimen (219).

Clinical Applications

Etravirine's clinical utility is limited to treatment-experienced patients, but its tolerability and side-effect profile make it an appealing treatment option in combination with other active agents, particularly for those retaining etravirine susceptibility after failing other regimens. Etravirine monotherapy demonstrated considerable potency and tolerability over 7 days in treatment-naïve individuals (220). In treatment-experienced individuals failing initial NNRTI regimens, etravirine has virologic activity against NNRTI-resistant virus (217, 221). The addition of etravirine in highly treatment-experienced individuals results in an increased proportion of individuals achieving sustained virologic suppression to <50 copies/ml and greater increases in CD4 cell counts than with placebo (211, 212). The efficacy of etravirine in highly treatment-experienced patients is adjunctive and often difficult to separate from the durable virologic response of other fully active drugs included in the new, combination regimens (210).

Rilpivirine

Rilpivirine [4-((4(4-((1E)-2-cyanoethenyl)-2,6-dimethylphenyl)amino)-2-pyrimydinyl)amino)benzonitrile; TMC 278, Edurant] (Figs. 1 and 4; Tables 1 and 3) is a diaryl-pyrimidine analog that demonstrates conformational "flexibility" enabling it to bind to the hydrophobic pocket of reverse transcriptase of both wild-type and drug-resistant variants (222). The IC_{50} is <1 nM (0.37 ng/ml). It is available as 25-mg tablets or in fixed-dosed combinations with emtricitabine and TDF (Complera) or with emtricitabine and TAF (Odefsey). A nanosuspension formulation of rilpivirine is under study, in combination with cabotegravir, and is being considered for both maintenance treatment in HIV positive persons and prevention in HIV negative persons (1, 63).

Pharmacology

The oral bioavailability of rilpivirine is unknown but peak concentrations of ∽160 ng/ml are achieved within 4–5 hours. Administration in the fasting state or with only a nutritional supplement decreases the AUC by 40–50%, so rilpivirine should be administered with meals. Rilpivirine is highly protein bound (>99%), and CSF concentrations average only 1.4% of plasma concentrations (223). Rilpivirine is metabolized by CYP3A. The terminal $t_{1/2}$ is about 50 h allowing for once-daily oral dosing. Rilpivirine-LA is a nanosuspension which is administered intramuscularly and demonstrates a $t_{1/2}$ of 33–35 hours (224).

Adverse Effects

The most common adverse effects associated with rilpivirine are headache, insomnia, and rash. In clinical trials, the frequency of neurologic and psychiatric adverse events, lipid elevations, and rash were less with rilpivirine compared to efavirenz. Severe skin and hypersensitivity reactions are still of concern. Liver function tests should be monitored in patients with underlying liver disease. Higher doses of rilpivirine (75 mg daily) have been associated with a maximum QTcF interval-prolongation of ∽10.7 msec at 16 hours post-dosing. At therapeutic dosing (25 mg daily) rilpivirine has not been associated with a significant prolongation of the QTc interval, although some abnormalities were noted and several events had a delayed onset that may not have been observed in earlier studies. The drug is better avoided in individuals with QTc prolongation at baseline and in those likely to be prescribed other drugs that may confer this risk.

Drug Interactions

Rilpivirine is a substrate of the CYP3A4 isoenzyme. Exposure to rilpivirine is increased during coadministration with ritonavir, but no dosage adjustments of rilpivirine are recommended with boosted PIs. Rilpivirine concentrations are decreased by the coadministration with rifabutin. Due to increased gastric pH and impaired rilpivirine absorption, caution must be exercised with antacids, histamine 2-receptor antagonists (H2RA), and proton pump inhibitors. Antacids should be administered 2 hours before or 4 hours after a rilpivirine dose. H2RAs decrease rilpivirine AUC ∽76% and should be administered at least 4 hours after or 12 hours before a rilpivirine dose. Proton pump inhibitors are contraindicated.

Resistance

Rilpivirine has a somewhat low barrier to resistance, and, when compared to efavirenz-based regimens, those failing rilpivirine-based regimens were more likely to develop resistance mutations, although the role of tolerance and adherence needs to be taken into account in assessing how much pressure was placed on the virus (225). Mutations associated with rilpivirine resistance include L100I, K101E/P, E138A/G/K/Q/R, V179L, Y181C/I/V, Y188L, H211Y, F227C, and M230I/L. The K103N/R/S and V179D mutations do not confer resistance as single mutations but do when detected in the combinations L100I plus K103N/S or L100I plus K103R and V179D (28, 226). The E138A mutation and others may occur more frequently as natural polymorphisms in non-B subtype viruses (227). The M184I resistance mutation, which confers resistance to the NRTIs lamivudine and emtricitabine also potentiates rilpivirine resistance conferred by E138K and K101E (28, 228–230).

Clinical Applications

Rilpivirine is used in treatment-naïve patients with an HIV-1 RNA level <100,000 copies/ml. Virologic failure is more common when rilpivirine-based regimens are initiated in those with HIV-1 RNA levels >100,000 copies/ml, compared with those with lower HIV-1 plasma RNA levels and those receiving efavirenz-based regimens (225, 231, 232).

However, those on rilpivirine-based regimens have had fewer adverse events when compared to efavirenz-based regimens. Few data are available on treatment combinations other than rilpivirine plus tenofovir-emtricitabine. The restriction on using rilpivirine in those with high-baseline HIV-1 RNA levels, as well as the requirement that it must be taken with at least a 400-calorie meal and in the absence of acid-lowering agents, restrict its utility for many treatment-naïve patients. However, the small rilpivirine pill size or the fixed-dose combination rilpivirine/tenofovir/emtricitabine makes it an appealing option.

INVESTIGATIONAL NNRTIS

A number of investigational NNRTI compounds continue through preclinical and clinical development. These agents are typically characterized by activity against HIV-1 strains with K103N and Y181C mutations and possess improved safety profiles. Doravirine (MK-1439) is one in a new class of compounds called diarylpyrimidines whose 48-week efficacy data suggest comparable *in vivo* activity to efavirenz with HIV viral loads <100,000 copies/ml. Selected agents are listed in Table 2.

INTEGRASE STRAND TRANSFER INHIBITORS

HIV integrase is a virally encoded enzyme that is responsible for integrating the viral DNA into the genome of the host cell (233). Integration is a multistep event that involves processing of the viral DNA by removing the terminal dinucleotides, formation of the preintegration complex, and integration of the DNA strand into the cellular DNA. The enzyme incorporates viral DNA strands into the host chromosome through strand transfer. Integrase inhibitors belong to a class of antiretroviral agents that selectively inhibit the strand transfer function of the HIV integrase enzyme, thereby preventing integration and inhibiting HIV replication (234, 235).

Raltegravir

Raltegravir {N-[2-[4-[(4-fluorophenyl)methylcarbamoyl]-5-hydroxy-1-methyl-6-oxopyrimidin-2-yl]propan-2-yl]-5-methyl-1,3,4-oxadiazole-2-carboxamide; MK-0518, Isentress} (Figs. 1 and 5; Tables 1 and 4) was approved by the FDA in October 2007 at a dosage of 400 mg orally twice daily as the lead compound in this crucial drug class.

Pharmacology

Raltegravir is active against viruses resistant to all other classes of antiretrovirals, with an IC_{95} of 31 ± 20 nM against the H9IIIB variant of HIV-1 in human cell lines. The plasma C_{max} averages 4.94 μM 1 hour after a single dose of 200 mg in healthy volunteers (236), and levels in plasma decline below the limits of detection by 24 hours. Food increases the variability of raltegravir bioavailability, but the clinical significance is unknown and raltegravir can be administered without regard to food. In single and multiple dose-ranging studies in healthy and infected subjects, concentrations in serum declined in a biphasic manner, with an initial-phase elimination $t_{1/2}$ of approximately 1 h and a terminal-phase elimination $t_{1/2}$ of 7 to 12 hours. About 83% of raltegravir is protein bound; CSF concentrations have been estimated at 5.8% of plasma concentrations. The major mechanism of clearance of raltegravir in humans is by hepatic UGT1A1 to a glucuronidated metabolite excreted primarily in feces (51% of the dose) and urine (32% of the dose) (236). No dose adjustment appears necessary in patients with severe renal insufficiency.

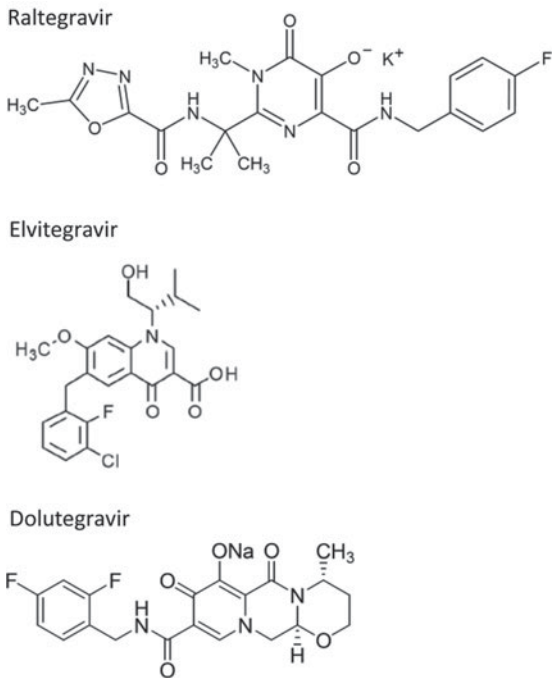

FIGURE 5. Chemical structures of HIV integrase inhibitors.

Adverse Effects

Raltegravir is generally well tolerated and does not seem to be associated with lipid abnormalities. Possible side effects include nausea, headache, dizziness, and fatigue. Symptomatic skeletal muscle toxicity including creatine phosphokinase elevations and rhabdomyolysis have been associated with raltegravir therapy (237, 238).

Drug Interactions

Raltegravir is eliminated primarily by the UGT1A1-mediated glucuronidation pathway; therefore, it may be subject to drug interactions when coadministered with UGT1A1 inducers or inhibitors (e.g., atazanavir, rifampin). Levels of raltegravir in plasma may be mildly increased during concomitant use with tenofovir, atazanavir with or without ritonavir, and omeprazole while raltegravir may be mildly decreased during concomitant use with efavirenz, etravirine, and tipranavir-ritonavir. However, no raltegravir dose adjustments are recommended during coadministration of these drugs. Rifampin significantly decreases raltegravir AUC by 40%, but increasing the dose of raltegravir to 800 mg b.i.d. compensates for this decrease (239). The impact of other UGT1A1 inducers (e.g., phenytoin) on the pharmacokinetics of raltegravir remains unknown.

Resistance

HIV-1 develops resistance to raltegravir with mutations in the active site of the integrase gene, and cross resistance between currently available integrase inhibitors, particularly between raltegravir and elvitegravir, is common for individuals failing either agent (28). Virologic failure was associated with mutations Q148H/K/R and N155H, which represent signature mutations that define two pathways of

TABLE 4 Integrase inhibitors: pharmacokinetic, major toxicity, and drug interaction characteristics

Agent	Oral bioavailability	Effect of food on AUC	$t_{1/2}$ (h)	Route of metabolism	Adjustment for hepatic and renal insufficiency	Major toxicities	Major drug interactions
Raltegravir	Unknown	Variable	9	Hepatic metabolism via UGT1A1; excretion: 51% feces, 32% urine	none	Well tolerated, no lipid abnormalities; possible nausea, headache, dizziness, fatigue, rare rhabdomyolysis	Aluminum hydroxide, magnesium salts: decreased levels of raltegravir UGT1A1 inhibitors (tenofovir, atazanavir, and omeprazole): increased levels of raltegravir UGT1A1 inducers (efavirenz, etravirine, rifapentine, rifabutin, and rifampin): decreased levels of raltegravir, requires increased dose of raltegravir
Elvitegravir	Unknown	Fasting decreases AUC by 50% (must be taken with food)	12.9[a]	CYP3A with hepatobiliary elimination	If combined with cobicistat, do not initiate if CrCl < 70ml/min, stop therapy if CrCl < 50ml/min; not recommended in severe hepatic impairment	Nausea, diarrhea	CYP3A inducers (carbamazepine, efavirenz, phenytoin, nevirapine, oral contraceptives, rifapentine, rifabutin, and rifampin): decreased levels of elvitegravir CYP3A inhibitors (azoles): increased levels of elvitegravir
Dolutegravir	Unknown	Food increases AUC by 33–66%	14	Hepatic metabolism via UGT1A1, small CYP3A contribution	No adjustments but not recommended for CrCl < 30ml/min or severe hepatic impairment	Well tolerated; nausea, diarrhea, insomnia, headache; no lipid abnormalities	CYP3A inducers (carbamazepine, etravirine, nevirapine, phenytoin, phenobarbital, carbamazepine, oxycarbazepine, St. Johns Wort): decreased dolutegravir levels, avoid coadministration; Increase to 50 mg bid if given with efavirenz, rifampin, or some ritonavir-based regimens; administer 4 hours before or 6 hours after cation containing antacids.

[a]If elvitegravir given with pharmacokinetic enhancer.

resistance. Each of these mutations is typically accompanied by one or more additional mutations in isolates derived from patients exhibiting virologic failure on raltegravir. The Q148H/K/R pathway consists of L74M plus E138A/K or G140A/S. The N155H pathway minor mutations are L74M, E92Q, or T97A, plus T97A, Y143R/H/C, G162K/R, V151I, or D232N (28, 240, 241). The F121 mutation is considered a major mutation and also confers some cross-resistance to dolutegravir and elvitegravir (242); G118R, though rare in those failing raltegravir, also confers resistance to raltegravir, elvitegravir, and dolutegravir (243). The presence of R263K, a minor mutation, confers 2- to 5-fold reductions in susceptibility to all currently approved integrase inhibitors but also conveys a loss of viral replicative capacity (28, 244).

Clinical Applications

Raltegravir has been evaluated as part of initial therapy in treatment-naïve individuals and as salvage therapy in individuals harboring a multidrug-resistant HIV strain. Tolerability and limited adverse effects have led to its inclusion as recommended initial therapy for treatment-naïve patients. Its twice-daily dosing has made it less appealing for some patients when compared with single-tablet regimens, but data suggest that a once-daily 1200-mg dose is noninferior to twice-daily dosing (245). Compared with efavirenz, in combination with tenofovir and lamivudine, raltegravir demonstrates higher rates of early virologic suppression to <50 copies/ml; at 48 weeks, however, comparable rates of virologic suppression and mean changes in CD4 cell counts (144 to 221 cells/mm^3) are seen in both groups (246–248).

When used in combination with optimized background therapy in treatment-experienced individuals with evidence of resistance to at least one drug in each of the three classes, raltegravir has resulted in as much as a 2-$\log_{10}$ decrease in week 24 HIV-1 RNA levels compared to optimized background therapy alone. Significantly greater proportions of patients in the raltegravir treatment arm achieved HIV-1 RNA levels of <50 copies/ml. Higher doses of the drug resulted in greater CD4 cell recovery (249). Favorable responses have been sustained at 48 weeks (249), thus leading to the selection of raltegravir at 400 mg orally twice daily as the dosage to take forward in clinical development.

In two pivotal studies, week 48 data confirmed the superior potency and durability of raltegravir compared to placebo, each in combination with an optimized background therapy, in individuals with triple-class resistant virus: 64% in the raltegravir arm achieved viral suppression to <50 copies/ml, compared to 34% in the placebo arm (250, 251). The availability of raltegravir has contributed substantially to achieving full virologic suppression (i.e., plasma HIV-1 RNA level of <50 copies/ml) in highly treatment-experienced patients with multidrug-resistant virus.

Elvitegravir

Elvitegravir {6-[(3-chloro-2-fluorobenzyl]-1-[(2S)-1-hydroxy-3-methylbutan-2-yl]-7-methoxy-4-oxo-1,4-dihydroquinoline-3-carboxylic acid; GS-9137, JTK-303, Vitekta} (Figs. 1 and 5; Tables 1 and 4) is a modified quinolone antibiotic that inhibits HIV-1 integrase. It was approved in 2012 as a fixed-dose combination with cobicistat, emtricitabine, and TDF (Stribild). It is now also available in combination with the same drugs except for the substitution of TAF (Genvoya) for the older disoproxil fumarate component. It is also available as a standalone agent in 85-mg and 150-mg tablets. Dosing is based on concomitant antiretrovirals. Elvitegravir inhibits strand transfer with an IC_{50} of 7.2 nM (3.2 ng/ml) and has an EC_{50} of about 0.9 nM (0.4 ng/ml) in HIV-1 infection assays. Elvitegravir is also effective against HIV-2 (252). In a 10-day dose-finding monotherapy study in treatment-naïve and -experienced patients with HIV-1 RNA levels between 10,000 and 300,000 copies/ml, elvitegravir resulted in a ≥1.0 $\log_{10}$ reduction of HIV-1 RNA (253).

Pharmacology

Elvitegravir achieves a C_{max} concentration of ∽1.5 μg/ml after a 150-mg dose with ritonavir approximately 4 hours post dose. Fasting decreases the elvitegravir AUC by 50% and thus must be taken with food. Protein binding is very high (>98%). Elvitegravir is metabolized by CYP450-mediated oxidation and glucuronidation (UGTs 1A1 and 1A3) with subsequent hepatobiliary elimination. The terminal $t_{1/2}$ is about 8.7 h when administered in combination with the pharmacoenhancers ritonavir or cobicistat.

Adverse Effects

The most common adverse effects associated with elvitegravir are nausea and diarrhea.

Drug Interactions

The CYP3A isoenzyme pathway is responsible for the metabolism of elvitegravir, and thus, drugs that induce or inhibit this pathway will decrease or increase elvitegravir levels, respectively. Ritonavir and cobicistat are used as pharmacoenhancers of elvitegravir such that ritonavir 100 mg and cobicistat 150 mg each provide near-maximal boosting of elvitegravir resulting in lower clearance, higher bioavailability, and longer $t_{1/2}$ (254, 255). When used with a PI, the dosing recommendations reflect the impact of the boosted PI on elvitegravir kinetics. Coadministration with lopinavir/ritonavir or atazanavir-ritonavir necessitates an elvitegravir dose of 85 mg daily while coadministration with boosted darunavir or fosamprenavir or tipranavir requires an elvitegravir daily dose of 150 mg.

Resistance

Integrase mutations leading to elvitegravir resistance in treatment-naïve and experienced patients include T66I/A/K, E92Q/G, T97A, F121Y, S147G, Q148H/K/R, N155H, and R236K (208, 256, 257). Mutations T66I, E92Q, F121Y, S147G, G148H/K/R, and N155H are all considered major mutations, those that substantially reduce elvitegravir susceptibility in the absence of other mutations. Cross-resistance is common between raltegravir and elvitegravir, and sequential use is not recommended (28, 83,258–260).

Clinical Applications

Elvitegravir, boosted with cobicistat, is used in a fixed-dose combination with emtricitabine and TAF or TDF as initial therapy for treatment-naïve patients (105). When compared with an atazanavir, ritonavir plus TDF-emtricitabine regimen, elvitegravir/cobicistat/emtricitabine/TDF had similar rates of virologic failure and a favorable side effect profile (261, 262). Elvitegravir also had similar efficacy and safety to raltegravir when combined with a ritonavir-boosted PI in treatment-experienced patients, over 60% of whom had two-class drug resistance (263).

Dolutegravir

Dolutegravir {(4R,12aS)-N-[(2,4-difluorophenyl)methyl]-7-hydroxy-4-methyl-6,8-dioxo-3,4,12,12a-tetrahydro-2H-pyrido[5,6]pyrazino[2,6-b][1,3]oxazine-9-carboxamide; GSK1349572, Tivicay} (Figs. 1 and 5; Tables 1 and 4) is the

most recently approved integrase inhibitor of HIV-1. It is effective in the presence of most raltegravir- and elvitegravir-associated mutations. It is available as 50-mg tablets and in combination with abacavir and lamivudine (Triumeq).

Pharmacology

Dolutegravir exhibits potent antiviral activity with a median IC_{50} of 1.07 nM against 9 wild-type isolates. It is administered without regard to food, although food increases the dolutegravir AUC by 33–66%. Dolutegravir trough concentrations exceed the IC_{90} of most viral strains by at least 5-fold (264). Dolutegravir is primarily hepatically metabolized by UGT1A1 with some contribution of CYP3A. The elimination $t_{1/2}$ is ~14 hours, negating the need for a pharmacoenhancer and allowing for once-daily dosing. Renal elimination accounts for <1% of a dose, but patients with severe renal impairment (CrCl <30 ml/min) have an AUC that is 40% less compared to healthy matched controls. The mechanism of these lower exposures is unknown, but it is thought to be clinically significant only in treatment-experienced patients.

Adverse Effects

Dolutegravir, as with other members in this class, is generally well tolerated. The most common adverse effects are nausea, diarrhea, insomnia, and headache. Effects on lipids are minimal and comparable to raltegravir. An increase in serum creatinine of ~0.11 mg/dl occurs in the first 4 weeks of treatment and remains stable through 48 weeks of treatment, due to the cobicistat component that mediates the inhibition of creatinine secretion by the organic cation transport system; there is no impact on glomerular filtration rate.

Drug Interactions

Potent inducers of CYP3A4 decrease dolutegravir concentrations, necessitating an increased dose or in some cases alternative agents. Coadministration with etravirine (without select boosted PIs), nevirapine, phenytoin, phenobarbital, carbamazepine, oxcarbazepine, and St. John's wort is not recommended. When dolutegravir is used concomitantly with efavirenz, fosamprenavir-ritonavir, tipranavir-ritonavir, and rifampin, dolutegravir should be dosed at 50 mg twice daily. Efavirenz and rifampin decrease the dolutegravir AUC to 43% and 46% of usual exposure, respectively, which can be overcome with twice-daily dosing. Dolutegravir absorption is adversely affected by cation-containing antacids, sucralfate, iron, calcium supplements, and buffered medications (decrease absorption ~75%) such that dolutegravir must be administered either 2 h before or 6 h after these medications.

Resistance

Dolutegravir has a relatively high barrier to resistance, and several mutations are required in combination to induce high-level resistance (28, 265–267). The major mutation, Q148H/R, occurs in patients failing raltegravir- or elvitegravir-based regimens but has minimal effect on dolutegravir susceptibility alone (83, 268). Dual mutation combinations with E138A/K or G140A/S can cause more than a 10-fold decrease in dolutegravir susceptibility (269). Other minor mutations associated with dolutegravir resistance, when combined with other mutations, include F121Y, N155H, and R263K (28). The majority of dolutegravir-associated resistance mutations also confer resistance to raltegravir and elvitegravir, making cross-resistance a problem.

Clinical Applications

Dolutegravir has been extensively studied in treatment-naïve patients, and its efficacy and tolerability resulted in its being recommended as one of the preferred components of initial therapy by treatment guidelines committees (1, 105). Once-daily dolutegravir is noninferior to twice-daily raltegravir in combination with either tenofovir-emtricitabine or abacavir-lamivudine in treatment-naïve patients (270). Dolutegravir plus abacavir-lamivudine has superior rates of virologic suppression compared to the fixed-dose combination efavirenz/TDF/emtricitabine and primarily because of treatment discontinuation with efavirenz (139, 271). Dolutegravir-based regimens are also superior to darunavir-ritonavir-based regimens because of more frequent treatment discontinuation with the PI regimen (272, 273).

Dolutegravir has also shown efficacy, tolerability, and a high barrier to resistance in trials for treatment-experienced patients. A phase IIb study assessing the activity of dolutegravir plus optimized background regimens in patients with raltegravir resistance found that twice-daily dolutegravir dosing was more effective than once-daily (269). Dolutegravir-based regimens are superior to raltegravir-based regimens in treatment-experienced patients with at least two-class drug resistance but no prior exposure to integrase inhibitors, demonstrating higher rates of virologic suppression and lower risk for development of resistance (274).

INVESTIGATIONAL INTEGRASE INHIBITORS

In the current era of increased use of integrase inhibitors, additional agents with a focus on potency and less frequent dosing are being developed (see Table 2). Bictegravir (GS-9883) has more favorable pharmacokinetics, comparable tolerability, and an improved resistance profile compared to other INSTIs, including dolutegravir. Bictegravir is being formulated as a combination product containing bictegravir/emtricitabine/TAF and is currently in phase 3 clinical trials. A novel agent in this class is cabotegravir (S/GSK1265744). Its structure is similar to that of dolutegravir with an elimination $t_{1/2}$ of ~40 hours following oral administration. Cabotegravir is also formulated in a long-acting injectable as nanoparticles resulting in an extended half-life of 21–50 days following a single dose that would potentially allow for bimonthly, intramuscular injections (275), but this is still under study. It is being developed for both the treatment and prevention of HIV-1 infection.

HIV PROTEASE INHIBITORS

Retrovirus replication requires virus-mediated proteolytic cleavage of *gag* and *gag-pol* polypeptide precursors mediated by the dimeric viral aspartyl protease. There are currently nine approved HIV PIs that share several characteristics: (i) are peptidomimetics; (ii) bind to the active site with a noncleavable scissile bond; (iii) have potent *in vivo* activity against HIV-1 when used in combination regimens; (iv) have *in vitro* activity against both HIV-1 and HIV-2; (v) have various degrees of cross-resistance that can be treatment limiting; and (vi) have pharmacological profiles that confer a significant potential for drug interactions with other antiviral and non-antiviral agents. For a number of agents in this class, this latter characteristic can be exploited to improve pharmacological profiles with "boosting" using either ritonavir or cobicistat. The unique resistance characteristics

(e.g., large number of mutations required to confer resistance) and antiviral efficacy of boosted PIs make them attractive choices for initial as well as salvage therapy (276, 277). Second-generation PIs such as tipranavir and darunavir present additional options for patients who are highly treatment-experienced and have HIV-1 strains resistant to other PIs.

Potential interactions with PIs may result from the coadministration of drugs that affect the CYP3A4 isozyme. Major drug interactions for PIs are listed in Table 5. Specific drugs and drug classes of this kind (e.g., astemizole and terfenadine, midazolam, triazolam, simvastatin, lovastatin, cisapride, and ergot alkaloids) may lead to severe toxicity and, therefore, are contraindicated in combination with CYP3A4-metabolized PIs. Conversely, inducers of the CYP3A4 isozyme (e.g., rifabutin and rifampin) can enhance PI clearance and lead to subtherapeutic levels.

Caution is required in administering PIs along with nonprescription herbal remedies. Since the currently available PIs are P-glycoprotein substrates and since constituents of St. John's wort are P-glycoprotein inducers, their coadministration should be avoided because therapeutic failure could result from reduced PI bioavailability.

Metabolic disorders have been increasingly recognized in the era of more potent antiretroviral therapy. Beginning in the late 1990s, case reports and studies began implicating PIs in causing abnormal glycemic control, insulin resistance, and increased triglyceride and cholesterol levels as soon as 14 days after treatment initiation. Accompanying body morphology changes have been described (278–280). These include fat distribution changes like truncal obesity, dorsocervical fat ("buffalo hump"), lipomatosis, and gynecomastia, as well as lipoatrophy from the buttocks, face, and extremities. The lipoatrophic changes have also been attributed to nucleoside analog therapy, and attempts to dissect the causality of the morphological changes continue. Lipodystrophic changes may be accompanied by insulin resistance and hypertriglyceridemia.

The PI era began in earnest in the mid-1990s with the reporting of the virologic and clinical benefits of indinavir-zidovudine-lamivudine and ritonavir (281–283). The fortunate parallel development and implementation of plasma HIV-1 RNA assays facilitated the performance of clinical trials and were a boon to clinicians and patients as monitoring tools (284, 285). The era of truly potent, fully suppressive antiretroviral therapy (ART) began in 1996 and sparked further drug development to make regimens simpler, more effective, and less toxic. Saquinavir was the first PI approved but was hampered by poor absorption. Thus, the *in vivo* potency of this class of drugs was realized by full-dose ritonavir and indinavir that resulted in durable virologic suppression; reductions in HIV-related disease progression and death followed. Saquinavir and indinavir are now little used, and ritonavir's use is restricted as a low-dose pharmacoenhancer of other antiretrovirals. The next wave of PI development brought nelfinavir, amprenavir, fosamprenavir, and lopinavir/ritonavir, with the latter becoming an important part of therapeutic regimens worldwide. Thus, this section will detail the characteristics of lopinavir/ritonavir, atazanavir, tipranavir, and darunavir. The other approved but currently little used PIs, are discussed in the third edition of this textbook.

Lopinavir (Coformulated with Ritonavir)

Lopinavir {(2S)-N-[(2S,4S,5S)-5-[[2-(2,6-dimethylphenoxy) acetyl]amino]-4-hydroxy-1,6-diphenylhexan-2-yl]-3-methyl-2-(2-oxo-1,3-diazinan-1-yl)butanamide; Kaletra} is structurally related to ritonavir (Figs. 1 and 6; Tables 1 and 5). For pharmacological enhancement, it is formulated in a fixed-dose combination with ritonavir in a tablet containing 200 mg of lopinavir and 50 mg of ritonavir. The liquid formulation contains 80 mg of lopinavir and 20 mg of ritonavir per ml; the capsule formulation has been discontinued. The more recent formulation offers lower pill burden, less drug level variability, enhanced tolerability, and heat stability. In the absence of significant mutations, drug interactions, and pregnancy, ritonavir-boosted lopinavir is approved for once-daily dosing.

Lopinavir was designed primarily to reduce the dependence of PI activity on the drug molecule interacting with the isopropyl side chain of the V82 amino acid of the target HIV-1 protease. As a result, lopinavir exhibits more potent activity against both wild-type and mutant HIV-1 strains than does ritonavir. The IC_{50} of lopinavir is approximately 10-fold lower than that of ritonavir. The mean IC_{50} of lopinavir ranges from 4 to 11 nM (0.003-0.007 μg/ml) for HIV-1 subtype B clinical isolates.

Pharmacology

In healthy volunteers, a 400-mg dose of lopinavir alone only transiently produces plasma drug levels of > 0.1 μg/ml due to rapid metabolism. To compensate for these low levels, lopinavir was developed with pharmacokinetic enhancement by ritonavir to inhibit lopinavir metabolism. The resulting lopinavir/ritonavir combination has an $AUC_{0\text{-}24h}$ that is 77-fold greater than that of lopinavir alone, and it has a plasma elimination $t_{1/2}$ of about 10 hours. Women in their second and third trimester of pregnancy have 40% lower trough concentrations of lopinavir compared to post-partum; this difference is considered clinically significant for women who harbor viruses with reduced susceptibility. Lopinavir is more than 98% protein bound, but the CSF penetration of lopinavir/ritonavir results in levels which exceed the IC_{50} for wild-type HIV-1. Lopinavir is metabolized by CYP3A4; lopinavir metabolism results in the formation of at least 13 inactive oxidative compounds.

Adverse Effects

Gastrointestinal adverse effects (diarrhea, abdominal discomfort, and nausea) are the most commonly reported adverse effects. These symptoms typically occur in the initial 2 months of therapy and subsequently diminish in frequency. Lopinavir/ritonavir has a greater impact on cholesterol, particularly on triglycerides, than do atazanavir-ritonavir and saquinavir-ritonavir (286); some of these abnormalities may be reversible upon switching to another PI (e.g., atazanavir or a NNRTI). Like other PIs, lopinavir/ritonavir may contribute to abnormalities of body fat redistribution.

Diarrhea has been reported with a greater frequency at the 800-mg/200-mg once-daily dosing schedule approved for antiretroviral-naïve patients than with conventional dosing (17% vs. 5%, respectively) (287). Hepatotoxicity and pancreatitis have been reported. Changes in the lipid profile typically occur in the first month of treatment (288). Prolongation of the QT and PR intervals are of concern and caution is necessary in patients at risk.

Drug Interactions

Lopinavir/ritonavir is an inhibitor of CYP3A4. Efavirenz reduces the levels of lopinavir/ritonavir due to its CYP450-inducing activity; therefore, the dose of lopinavir/ritonavir should be increased when used in combination with efavirenz.

TABLE 5 Commonly used protease inhibitors: pharmacokinetic, major toxicity, and drug interaction characteristics

Agent	Oral bioavailability	Effect of food on AUC	$t_{1/2}$ (h)	Route of metabolism	Adjustment for hepatic and renal insufficiency	Major toxicities	Major drug interactions
Lopinavir/ ritonavir	Unknown	Increased by 48–130%	5–6	CYP3A4 inhibitor and substrate	Hepatic: no Renal: no	Diarrhea, nausea, vomiting, elevated transaminases, hyperlipidemia, hypertriglyceridemia, fat redistribution, metallic taste, asthenia, potential for increased bleeding in patients with hemophilia	Decreased methadone levels
Tipranavir	Unknown	None	6	CYP3A4 inducer and substrate; combined with ritonavir CYP3A4 inhibitor and CYP2D6 inhibitor; potent inducer of P-glycoprotein	Hepatic: use with caution in moderate to severe impairment Renal: no	Rash (especially patients with sulfonamide allergy); nausea; vomiting; diarrhea; hepatotoxicity, including hepatic decompensation (especially with underlying liver disease); hyperlipidemia; hypertriglyceridemia; fat redistribution; case reports of intracranial hemorrhage; potential for increased bleeding in patients with hemophilia	Clarithromycin, azole antifungals: increased tipranavir levels Erectile dysfunction agents: increased levels by tipranavir-ritonavir Lipid-lowering agents (atorvastatin, simvastatin): increased levels by tipranavir-ritonavir and increased potential for myopathy or rhabdomyolysis Amiodarone: increased potential for serious or life-threatening cardiac arrhythmias Rifampin and St. John's wort: decreased tipranavir levels Etravirine, zidovudine: decreased levels by tipranavir
Atazanavir	Unknown	Increased	7	CYP3A4 inhibitor and substrate	Hepatic: yes Renal: no	Prolonged PR interval and first-degree AV block (asymptomatic), indirect hyperbilirubinemia, abnormal liver enzymes, hyperglycemia, fat redistribution, potential for increased bleeding in patients with hemophilia	Proton pump inhibitors decrease atazanavir absorption, and concurrent administration is contraindicated; other antacids and H2 blockers should be used with caution Efavirenz: decreased atazanavir levels; addition of ritonavir may compensate for the interaction Etravirine: decreased atazanavir levels and etravirine levels increased by atazanavir; coadministration not recommended

(*Continued*)

TABLE 5 Commonly used protease inhibitors: pharmacokinetic, major toxicity, and drug interaction characteristics (*Continued*)

Agent	Oral bioavailability	Effect of food on AUC	$t_{1/2}$ (h)	Route of metabolism	Adjustment for hepatic and renal insufficiency	Major toxicities	Major drug interactions
Darunavir	37%; 82% with ritonavir	Increase	15 (with ritonavir)	CYP3A4 inhibitor and substrate	Hepatic: yes Renal: no	Rash; diarrhea; nausea; headache; cold-like symptoms, including runny nose or sore throat; elevated transaminases; hyperlipidemia; hypertriglyceridemia	Lipid-lowering agents, calcium channel blockers, antiarrhythmics, erectile dysfunction agents, clarithromycin, tenofovir: increased levels by darunavir or darunavir-ritonavir Rifampin: decreased darunavir levels Rifabutin: increased levels by darunavir, requires reduced rifabutin dose Caution with lopinavir-ritonavir or efavirenz (decreased darunavir levels and increased coadministered drug levels) Maraviroc: increased levels; adjust dose to 150 mg b.i.d. when combined with darunavir-ritonavir

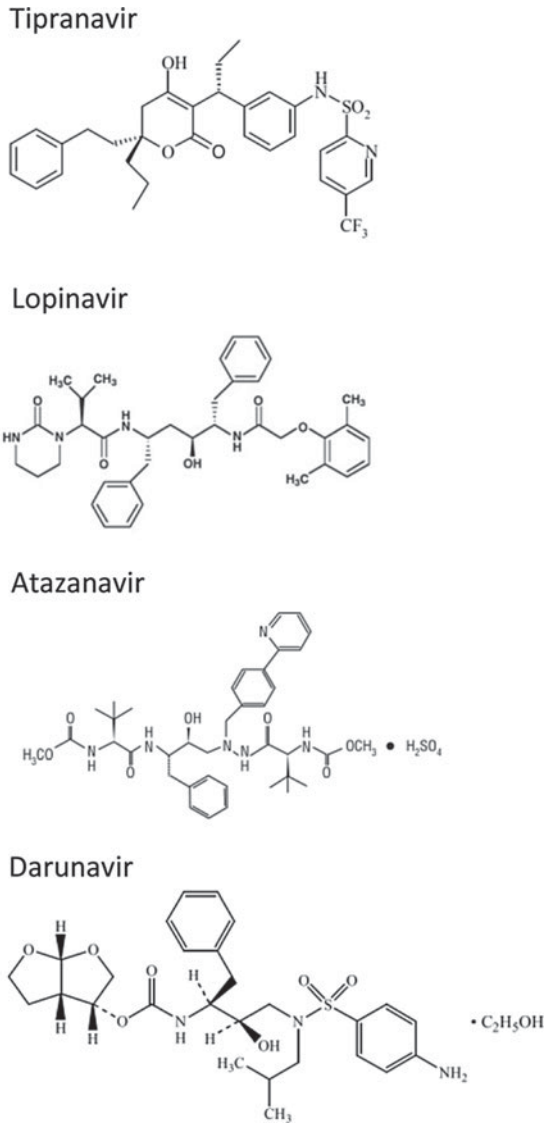

FIGURE 6. Chemical structures of HIV protease inhibitors.

Tipranavir decreases lopinavir levels significantly and should not be coadministered. Rifampin decreases lopinavir levels by 75%, an effect that may be overcome by administering 800 mg lopinavir/200 mg ritonavir twice daily or 400 mg lopinavir/400 mg ritonavir twice daily (289). Lopinavir has been shown to significantly reduce methadone levels, resulting in opiate withdrawal symptoms.

Resistance

Coformulated lopinavir/ritonavir demonstrates a high genetic barrier to resistance, requiring six or more mutations for reduced virologic response (290, 291). At least 10-fold reductions in susceptibility are needed before a change in virologic response is observed, and ≥60-fold reductions in susceptibility are required before the activity of a standard dose of the drug is no longer sufficient to reduce plasma HIV-1 RNA levels by half a log. The characterization of *in vitro*-selected lopinavir-resistant HIV-1 strains reveals a sequential accumulation of mutations in the protease gene that include L10F/I/R/V, K20M/R, L24I, V32I, L33F, M46I/L, I47V/A, I50V, F53L, I54V/L/A/M/T/S, L63P, A71V/T, G73S, L76V, V82A/F/T/S, I84V, and L90M (28), with major mutations being those at positions 32, 47, 76, and 82. The V32I and I47A mutations are associated with high-level resistance (292–294). In lopinavir/ritonavir-naïve patients with wild-type virus who experience virologic failure, emergence of resistance mutations is rare (293, 294).

Clinical Applications

Lopinavir/ritonavir is no longer a preferred regimen for treatment-naïve patients because of its high pill burden (four per day) and the side effects associated with its higher doses of ritonavir. However, it has a robust track record of safety and efficacy, a relatively high barrier to resistance, and is more affordable than darunavir. Thus, it is widely used in resource-constrained settings, particularly as the linchpin for second-line therapy following virologic failure on an NNRTI-based regimen (295). A direct comparison of lopinavir/ritonavir, either once or twice daily, to darunavir-ritonavir once daily, both combined with tenofovir-emtricitabine in treatment naïve patients, showed superior virological response and a more favorable adverse events profile with fewer gastrointestinal toxicities in the darunavir-ritonavir arm (296). Better virologic responses were also seen with an efavirenz-based regimen than with a lopinavir/ritonavir-based regimen, but better CD4 cell responses and less resistance were seen following virologic failure with lopinavir/ritonavir plus two NRTIs (197). Atazanavir-ritonavir and twice daily lopinavir/ritonavir, each in combination with tenofovir-emtricitabine, show similar efficacies, although atazanavir-ritonavir confers better lipid profiles and fewer gastrointestinal side effects (297, 298). Lopinavir/ritonavir twice daily plus lamivudine is one of the few recommended alternative regimens for treatment-naïve individuals that use fewer than two NRTIs (299).

Lopinavir/ritonavir also is used in salvage therapy. In combination with NNRTIs and NRTIs, lopinavir/ritonavir has resulted in virologic suppression to <400 copies of HIV RNA/ml in patients failing to respond to their initial or multiple PI regimens (300). In patients failing an NRTI plus NNRTI-based first-line regimen, the combination of raltegravir plus lopinavir/ritonavir is noninferior to lopinavir/ritonavir plus two or three NRTIs with respect to the proportion of patients with plasma HIV-1 RNA levels under 200 copies/ml (301). In patients with prior failure on a PI-based regimen, lopinavir/ritonavir demonstrates an efficacy comparable to that of ritonavir-boosted atazanavir for suppression of plasma HIV-1 RNA levels to <50 copies/ml (302). The greatest benefit has been seen in patients with ≥4 major protease mutations at baseline (303). In lopinavir-naïve, treatment-experienced patients, the more recently introduced PIs such as darunavir-ritonavir or tipranavir-ritonavir proved noninferior to lopinavir/ritonavir treatment (304). Once-daily dosing is not recommended in PI-experienced patients.

While some data support the use of lopinavir/ritonavir monotherapy following a fully suppressive regimen, its use as simplification therapy is not recommended (305, 306). Two studies found that large proportions of patients on lopinavir/ritonavir monotherapy after initial virologic suppression experienced suboptimal virologic responses compared to those who continued use of combinations (307, 308). Lopinavir/ritonavir monotherapy also led to less virologic suppression than combination lopinavir/ritonavir plus tenofovir-emtricitabine in patients failing NNRTI-based regimens (309).

TABLE 6 Fixed-dose single-table regimens. All are given as one tablet orally, once daily.

Agent	Components	Comments
Atripla	Efavirenz 600 mg	Alternative regimen for treatment-naïve patients because of association with suicidality in some studies
	Tenofovir disoproxil fumarate 300 mg	Take on an empty stomach
	Emtricitabine 200 mg	Nighttime dosing recommended to reduce impact of associated drowsiness, dizziness, and other central nervous system side effects.
Complera	Rilpivirine 25 mg	Alternative regimen for treatment naïve patients
	Tenofovir disoproxil fumarate 300 mg	Only for patients with pre-treatment HIV RNA level < 100,000 copies/ml and CD4+ cell count > 200 cells/mm^3
	Emtricitabine 200 mg	Must be taken with a meal (≥ 400 calories)
Genvoya	Elvitegravir 150 mg	Recommended regimen for treatment-naïve patients
	Cobicistat 150 mg	Only for patients with pre-treatment estimated creatinine clearance ≥ 30 ml/min
	Tenofovir alafenamide 10 mg	
	Emtricitabine 200 mg	
Odefsey	Rilpivirine 25 mg	Must be taken with a meal (≥ 400 calories)
	Tenofovir alafenamide 25 mg	Only for patients with pre-treatment HIV RNA level < 100,000 copies/ml
	Emtricitabine 200 mg	Only for patients with pre-treatment estimated creatinine clearance ≥ 30 ml/min
Stribild	Elvitegravir 150 mg	Recommended regimen for treatment-naïve patients
	Cobicistat 150 mg	Only for patients with pre-treatment estimated creatinine clearance ≥ 70 ml/min
	Tenofovir disoproxil fumarate 300 mg	
	Emtricitabine 200 mg	
Triumeq	Dolutegravir 50 mg	Recommended regimen for treatment-naïve patients
	Abacavir 600 mg	Only for patients who have tested negative for HLA-B*5701
	Lamivudine 300 mg	

Tipranavir

Tipranavir {(N-[3-[(1R)-1-[(2R)-6-hydroxy-4-oxo-2-(2-phenylethyl)-2-propyl-3,4-dihydro-2H-pyran-5-yl]propyl]-phenyl]-5-(trifluoromethyl)-2-pyridinesulfonamide; Aptivus} is a nonpeptidic PI belonging to the class of 4-hydroxy-5,6-dihydro-2-pyrone sulfonamides (Figs. 1 and 6; Tables 1 and 5). It is available as a 250-mg soft-gelatin capsule co-administered with ritonavir.

Tipranavir is active against a wide range of drug-resistant isolates and has a mean IC_{50} of 0.14 mg/l for highly multidrug-resistant clinical isolates (310). Potential mechanisms of its enhanced potency against PI-resistant virus include the ability to bind to the active site of the HIV protease with fewer hydrogen bonds, thereby creating greater flexibility at the active site (310) or strong hydrogen bonding interaction with the amide backbone of the protease active-site Asp30 (311, 312).

Pharmacology

Maximum mean tipranavir concentrations of 47 to 57 mg/l are achieved within 3 hours post-dosing. Food does not impact tipranavir absorption. Tipranavir is > 99% protein bound with a plasma elimination $t_{1/2}$ of 6 hours. As tipranavir is a substrate for CYP3A4, boosting with ritonavir is necessary to achieve a desirable pharmacodynamics profile. Ritonavir increases tipranavir concentrations 24- to 70-fold. To achieve adequate concentrations, tipranavir 500 mg must be coadministered with 200 mg of ritonavir twice daily. The higher dose is required because tipranavir is both a substrate and potent inducer of P-glycoprotein and may initially induce its own metabolism (313, 314). Tipranavir use is contraindicated in patients with moderate to severe hepatic impairment.

Adverse Effects

The most common adverse effects associated with tipranavir are predominantly gastrointestinal and include diarrhea, dyspepsia, abdominal distention, and pancreatitis. In two large clinical trials (RESIST 1 and RESIST 2) of more than 1,400 patients, which included patients coinfected with HBV or HCV but with stable liver enzymes at baseline, hepatic and lipid-related adverse effects occurred with greater frequency in the tipranavir-ritonavir arm but were rarely a cause for drug discontinuation. Hepatitis virus coinfection and a baseline CD4 cell count of > 200 cells/mm^3 were predictors of liver function abnormalities (315). Cases of clinical hepatitis and hepatic decompensation in patients with chronic HBV or HCV infection who received tipranavir-ritonavir have been reported.

Ritonavir-boosted tipranavir was linked to cases of intracranial hemorrhage, sometimes fatal, but a direct causative effect has not been proven, and many of the patients had other contributing risk factors. *In vitro* and *in vivo* data suggest that tipranavir can inhibit platelet aggregation and thus should be used with caution in patients receiving antiplatelet agents, anticoagulants, supplemental high doses of vitamin E, or who are at risk of increased bleeding after trauma or surgery (316).

Drug Interactions

Tipranavir both inhibits and induces the CYP450 enzyme system, and when tipranavir is used in combination with ritonavir, the net effect is inhibition of the CYP3A4 enzyme. Tipranavir is also an inducer of the P-glycoprotein transporter. Thus, tipranavir may alter the concentrations of many other drugs metabolized by these pathways (Table 4). Tipranavir decreases the concentrations of atazanavir,

fosamprenavir, lopinavir/ritonavir, and saquinavir and nucleoside analogs like abacavir, zidovudine, and didanosine. Coadministration of tipranavir-ritonavir with enfuvirtide may affect the volume of distribution and elimination $t_{1/2}$ of tipranavir, thereby resulting in a 45% increased C_{min} of tipranavir, although this interaction does not appear to result in an increased risk of hepatotoxicity.

Resistance

Tipranavir retains activity against many HIV-1 isolates with resistance to previously approved PIs such as nelfinavir, fosamprenavir, atazanavir, and lopinavir/ritonavir (310). Ninety percent of highly resistant clinical isolates (>10-fold resistance to three or more PIs) remain susceptible to tipranavir, and only 2% have >10-fold resistance to tipranavir (310); durable virologic responses are associated with a tipranavir resistance score of ≤2 at baseline (42). Major mutations include codons I47V, Q58E, T74P, V82L/T, N83D, and I84V (28). Minor mutations that correlate with a decreased response to tipranavir-ritonavir therapy in treatment-experienced patients are L10V, L33F, M36I/L/V, K43T, M46L, I54A/M/V, H69K/R, and L89I/M/V.

Clinical Applications

Tipranavir is approved for use, in combination with ritonavir, for treatment-experienced patients with resistance to more than one other PI (42). However, drug interactions, the need for 200 mg twice daily of ritonavir, the pill burden, and the possibilities of serious hepatotoxicity and perhaps intracranial hemorrhage, have led many providers to choose options other than tipranavir for treatment-experienced patients (105). Its use has been limited to the rare patient with darunavir resistance and with maintained susceptibility to tipranavir.

Atazanavir

Atazanavir {methyl N-[(2S)-1-[2-[(2S,3S)-2-hydroxy-3-[[(2S)-2-(methoxycarbonylamino)-3,3-dimethylbutanoyl]amino]-4-phenylbutyl]-2-[(4-pyridin-2-ylphenyl)methyl]hydrazinyl]-3,3-dimethyl-1-oxobutan-2-yl]carbamate; BMS-232632, Zrivada, Reyataz} (Figs. 1 and 6; Tables 1 and 5) is an azapeptide aspartyl PI. It is available in capsule formulations of 150, 200, and 300 mg and 50-mg oral powder packets and as a fixed-dose combination with cobicistat (Evotaz).

Atazanavir has activity against laboratory and clinical HIV-1 isolates grown in peripheral blood mononuclear cells and macrophages (IC_{50}, 2 to 5 nM) and variable activity against HIV-2 isolates (1.9 to 32 nM). Clinical isolates that are resistant to first-generation PIs may remain susceptible to atazanavir. However, isolates with high-level resistance, and hence, a larger number of mutations in the protease gene, are more likely to be associated with resistance to atazanavir.

Pharmacology

Atazanavir has rapid absorption that requires food and gastric acid; coadministration of a single dose with a light meal increases the AUC by 70%. A 400-mg once-daily dose attains mean steady-state C_{max} of 3.2 μg/ml and AUC of 22.3 μg*h/ml, well above the protein-binding-adjusted IC_{90}. Atazanavir is 86% protein bound and has a plasma elimination $t_{1/2}$ of approximately 7 hours. It is metabolized by hepatic CYP3A4 with only 13% of unmetabolized drug excreted in the urine. Atazanavir can be boosted by ritonavir 100 mg daily or cobicistat 150 mg daily.

The daily dose should be reduced to 300 mg daily in patients with moderate hepatic impairment due to a 46% increase in AUC. It is not recommended in patients with severe hepatic impairment, and boosted atazanavir is not recommended in any degree of hepatic impairment. The CSF-to-plasma ratio is 0.002 to 0.02, comparable to that of indinavir.

Adverse Effects

The most prominent adverse effect of atazanavir is reversible indirect hyperbilirubinemia, typically occurring in the first 60 days of treatment (Table 4). It is more frequent in persons with the UGT1A1-28 genotype or the CC genotype of the 3435C→T polymorphism in the multidrug resistance (MDR1) gene (317–320). Patients with Gilbert and Crigler-Najjar syndromes harbor polymorphisms in the UGT1A1 gene and demonstrate higher levels of indirect bilirubin after treatment with either indinavir or atazanavir than do untreated patients. Prolongation of the PR and QTc intervals has been noted. A case of torsade de pointes has been reported (321). Caution is advised when coadministering atazanavir with other drugs that cause PR interval prolongation.

Nephrolithiasis due to drug crystals may occur with use of ritonavir-boosted or unboosted atazanavir (322). Lipid abnormalities are slightly more pronounced in patients receiving ritonavir-boosted atazanavir than in those receiving atazanavir alone, but both regimens still have less of an impact on lipids than lopinavir/ritonavir and efavirenz.

Drug Interactions

Atazanavir is a substrate and inhibitor of CYP3A and UGT1A1 and a weak inhibitor of CYP2C8. Therefore, atazanavir should not be coadministered with agents with narrow therapeutic windows that are substrates of these isoenzymes (Table 4). Atazanavir, however, does not appear to induce its own metabolism. Drugs that induce or inhibit CYP3A activity may decrease or increase, respectively, plasma concentrations of atazanavir. Coadministration with tenofovir decreases serum atazanavir concentrations and increases concentrations of tenofovir (323); addition of low-dose ritonavir compensates for this interaction. Efavirenz decreases atazanavir levels and a higher boosted dose of atazanavir (400 mg with 100 mg ritonavir) is recommended. Atazanavir requires acidic gastric pH for dissolution; therefore, administration with proton pump inhibitors, which raise the gastric pH, significantly interferes with the absorption of atazanavir and can cause subtherapeutic serum atazanavir levels. H2 receptor antagonists may be an alternative if atazanavir is administered simultaneously or ≥10 hours after the H2 receptor antagonist.

Resistance

In vitro, accumulation of the mutations I50L, N88S, I84V, A71V, and M46I decreases susceptibility to atazanavir 93- to 183-fold. Changes are also observed at the protease cleavage sites following drug selection. In PI-naïve patients failing unboosted atazanavir, I50L is the most commonly observed mutation (324), but it occurs less frequently in patients receiving ritonavir-boosted atazanavir (28). Recombinant viruses containing the I50L mutation display increased *in vitro* susceptibility to other PIs (fosamprenavir, indinavir, lopinavir, nelfinavir, ritonavir, and saquinavir). A genotypic resistance score composed of 8 mutations (10F/I/V, G16E, L33I/F/V, M46I/L, D60E, I84V, I85V and L90M) has predicted that the occurrence of >3 of these mutations correlated with a reduced virologic response at 3 months, particularly when L90M is present (325). The presence of a

mutation at positions 46, 73, 84, or 90 at baseline is associated with a poorer virologic response (303). For unboosted atazanavir, the presence of 0, 1 to 2, 3, or ≥4 of the following mutations has been associated with 83, 67, 6, and 0% response rates: G16E, V32I, K20I/M/R/T/V, L33F/I/V, F53L/Y, I64L/M/V, A71I/T/V, I85V, and I93L/M.

Clinical Applications

Atazanavir as a once-daily administered PI with a favorable lipid profile and a higher genetic barrier to resistance than many NNRTI- or INSTI-based regimens is an alternative choice for both antiretroviral-naïve and -experienced individuals (105). In treatment-naïve patients, ritonavir-boosted atazanavir has efficacy similar to that of unboosted atazanavir but is associated with fewer virologic failures and a higher barrier to development of resistance. Atazanavir boosted with cobicistat appears pharmacokinetically similar to atazanavir-ritonavir (326). Unboosted atazanavir and atazanavir-ritonavir demonstrate potency comparable to that of efavirenz, each in combination with two nucleoside analogs (101, 327). Atazanavir-ritonavir plus tenofovir-emtricitabine is comparable in rates of virologic suppression to elvitegravir/cobicistat/tenofovir/emtricitabine for treatment-naïve patients at 96 weeks of treatment (328). When compared with darunavir-ritonavir or raltegravir plus tenofovir-emtricitabine, atazanavir-ritonavir plus tenofovir-emtricitabine shows a higher rate of discontinuation secondary to adverse events (329). Atazanavir-ritonavir has been used successfully in treatment-experienced patients (286), but it has not performed as well as tipranavir-ritonavir and darunavir-ritonavir in patients failing to respond to multiple PI regimens (41). Transitioning from boosted atazanavir to unboosted atazanavir plus abacavir-lamivudine is a safe strategy for virologically suppressed patients and improves bone and renal biomarkers (330, 331).

Darunavir

Darunavir {[(3aS,4R,6aR)-2,3,3a,4,5,6a-hexahydrofuro[2,3-b]furan-4-yl] N-[(2S,3R)-4-[(4-aminophenyl)sulfonyl-(2-hydroxy-2-methylpropyl)amino]-3-hydroxy-1-phenylbutan-2-yl]carbamate; Prezista, TMC-114}, is approved for treatment-naïve or -experienced persons (Figs. 1 and 6; Tables 1 and 5). It is available as 75-mg, 150-mg, 400-mg, 600-mg, and 800-mg tablets and a 100-mg/ml oral suspension as well as in a fixed-dose combination with cobicistat 150 mg (Prezcobix). Darunavir requires boosting to attain adequate systemic exposure, either with ritonavir or cobicistat. Darunavir exhibits activity against HIV-1 and HIV-2, with median IC_{50}s ranging from 1.2 to 8.5 nM (0.7 to 5.0 ng/ml) in PBMCs.

Pharmacology

Darunavir's oral bioavailability alone and after coadministration with a pharmacoenhancer averages 37% and 82%, respectively. The C_{max} in plasma is reached in 2.5 to 4 hours. Administration with food increases the darunavir AUC by 40%. Darunavir is approximately 95% protein bound. In the presence of ritonavir at 100 mg, darunavir has a rapid distribution and elimination phase followed by a slower elimination phase, yielding a terminal elimination $t_{1/2}$ of 15 hours. Darunavir is metabolized by hepatic CYP3A isoenzymes. No dosage adjustments are recommended for either renal or hepatic insufficiency. Considering darunavir's metabolism, caution is recommended in its administration to individuals with severe hepatic insufficiency.

Adverse Effects

The most common adverse effects are diarrhea, abdominal pain, and headache. Mild to moderate, typically self-limited maculopapular skin eruptions are uncommon (<5%), and severe skin rash, including erythema multiforme and Stevens-Johnson syndrome, is rare. Darunavir contains a sulfonamide moiety and should be used with caution in patients with a known sulfonamide allergy. There are reports of severe hepatitis, including fatal cases, occurring in approximately 0.5% of patients, particularly those with preexisting liver disease, including viral hepatitis.

Drug Interactions

Darunavir is both a substrate and an inhibitor of the CYP3A isoenzyme. As with other PIs, coadministration with drugs primarily metabolized by CYP3A may alter concentrations of darunavir and the other drug in plasma (Table 5).

Resistance

Major mutations associated with a diminished response to darunavir include I47V, I50V, I54M/L, L76V, and I84V, and minor mutations include V11I, V32I, L33F, T74P, and L89V (28, 332). In treatment-experienced patients, the presence of three or more of these mutations at baseline is correlated with a diminished virologic response to darunavir-ritonavir (41). In a clinical trial of darunavir-ritonavir as salvage therapy, the mutations V32I and I54L/M were present in more than 30% and 20% of isolates derived from patients with virologic failure, respectively, and the median darunavir IC_{50} of the virologic failure isolates was 21-fold higher at baseline and 94-fold at failure, compared to the reference virus strain. A higher probability of virologic response to darunavir-ritonavir occurs if the baseline darunavir change in the IC_{50} (compared with reference darunavir susceptibility) is less than 10-fold (41); 10- to 40-fold or >40-fold changes in phenotypic susceptibility to darunavir are a commonly used cutoff to differentiate between an intermediate or low likelihood of response to darunavir-ritonavir, respectively. Cross-resistance of darunavir with other PIs including atazanavir, lopinavir/ritonavir, and tipranavir is possible, but darunavir resistance mutations in patients failing other PIs is uncommon (333–335).

Clinical Applications

Darunavir, in combination with ritonavir or cobicistat pharmacoenhancement, is a robust backbone of first-line therapy for treatment-naïve patients and as salvage therapy for treatment-experienced patients. Darunavir-ritonavir has been shown to be superior to lopinavir-ritonavir, and equivalent to atazanavir-ritonavir or raltegravir-based regimens in treatment-naïve patients (296, 329). A darunavir-ritonavir plus two NRTIs regimen was inferior to a dolutegravir-plus-two-NRTI regimen, largely because of discontinuation from adverse events in the darunavir-based arms (272). Darunavir-ritonavir plus tenofovir-emtricitabine is also recommended for treatment-naïve patients who need to initiate antiretroviral therapy prior to HIV-1 genotype testing (105).

In treatment-experienced patients with evidence of resistance to PIs, darunavir-ritonavir in combination with nucleoside analogs provides higher rates of viral suppression to <50 copies/ml and immunologic recovery than in patients in the ritonavir-boosted PI comparator group (41, 336). Darunavir-ritonavir in patients with limited treatment experience (82% were susceptible to >4 PIs, all lopinavir/

ritonavir naïve) demonstrates noninferiority to lopinavir/ritonavir with respect to virologic suppression. Individuals with virologic failure on darunavir-ritonavir are more likely to maintain susceptibility to other PIs than individuals failing lopinavir/ritonavir regimens. Darunavir/ritonavir must be used in its twice-daily dosing format in patients with darunavir-associated resistance mutations, but can be used in a once-daily formulation for those with other PI mutations. The efficacy and durability of once-daily darunavir-ritonavir monotherapy and darunavir-ritonavir plus raltegravir as simplification strategies are also under consideration but are not recommended at this time (67, 337, 338).

INVESTIGATIONAL HIV-1 PROTEASE INHIBITORS

While PIs exhibit potent *in vitro* activity and the recent addition of second-generation PIs has marked a major advance in antiretroviral therapy, patients continue to experience virologic failure on these regimens. The search for newer agents with improved efficacy and toxicity profiles continues. Selected investigational PIs are described in Table 2.

PHARMACOENHANCERS

Ritonavir

Ritonavir {1,3-thiazol-5-ylmethyl N-[(2S,3S,5S)-3-hydroxy-5-[[(2S)-3-methyl-2-[[2-methylpropyl-[(2-propan-2-yl-1,3-thiazol-4-yl)methyl]carbamoyl]amino]butanoyl]amino]-1,6-diphenylhexan-2-yl]carbamate; Norvir} (Figs. 1 and 7; Table 1) is a C2-symmetric HIV PI that is used as a pharmacoenhancer of coadministered PIs. Ritonavir is available in tablet and oral solution formulations.

Pharmacology

Ritonavir was developed as a fully active PI but now is used solely as a pharmacoenhancer at lower doses, which improves its tolerance. Ritonavir is metabolized by hepatic microsomes, predominantly by the CYP3A4 and -3A5 isozymes and to a lesser extent by CYP2D6. In addition to being a hepatic microsomal substrate, ritonavir exhibits potent inhibitory activity against CYP3A4 and CYP2D6. It may also induce CYP3A, CYP1A2, CYP2C9, CYP2C19, and CYP2B6 and glucuronosyl transferase. Consequently, significant drug interactions are to be expected, and in the case of its interaction with other PIs, this can be exploited to boost their drug levels.

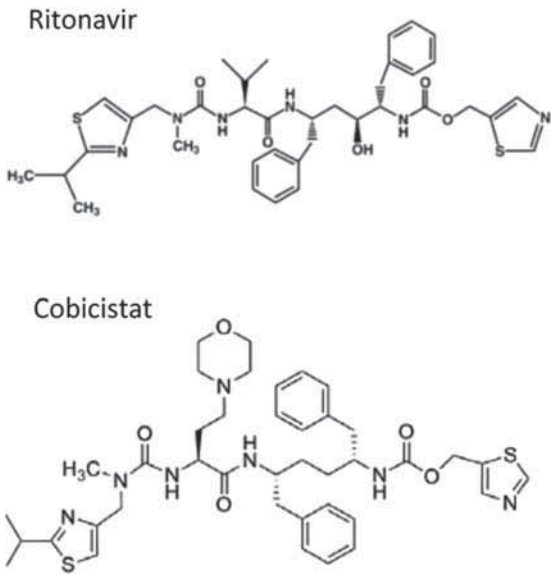

FIGURE 7. Chemical structures of pharmacoenhancers.

Adverse Effects

The most common adverse effects observed in clinical trials include gastrointestinal symptoms and paresthesias. Laboratory abnormalities typically observed with ritonavir at dosages of 400 mg twice daily or higher include hypercholesterolemia, hypertriglyceridemia, and elevated liver enzyme levels (339, 340). Ritonavir used in combination with other PIs is also associated with abnormalities in glucose metabolism and body fat distribution (278, 341–343).

Drug Interactions

See Pharmacology section above

Resistance

At the low dose employed for boosting of other PIs, ritonavir is thought to exert very limited to no anti-HIV-1 activity *in vivo* and therefore contributes to the emergence of PI-associated resistance mutations indirectly through the selective pressure exerted by the paired PI which is boosted.

Clinical Applications

Ritonavir is never used alone but is used as a pharmacoenhancer for other PIs. The commonly used PIs darunavir and lopinavir must be taken with ritonavir or cobicistat (for darunavir only as lopinavir is coformulated with ritonavir). Atazanavir can be used without ritonavir or cobicistat in treatment-naïve patients only. Ritonavir pharmacoenhancement of other PIs leads to improved antiretroviral regimens by (i) enhancing antiviral activity by elevating the trough levels of the coadministered PI well above the IC_{50} or IC_{90} associated with certain drug-resistant isolates and (ii) improving the ease of administration by diminished frequency of dosing and elimination of food effects. Intolerance to, or side effects from, ritonavir, even when used at a low dose, may be limiting.

Cobicistat

Cobicistat {1,3-thiazol-5-ylmethyl N-[(2R,5R)-5-[[(2S)-2-[[methyl-[(2-propan-2-yl-1,3-thiazol-4-yl)methyl]carbamoyl]-amino]-4-morpholin-4-yl)butanamido]-1,6-diphenylhexan-2-yl]carbamate; GS-9350, Tybost}(Fig. 1 and 7; Table 1) is an inhibitor of CYP3A metabolism that increases the concentrations of CYP3A substrates. Cobicistat has no antiviral activity of its own and is better tolerated than ritonavir. It is currently approved to be used in fixed-dose combinations with atazanavir and darunavir once daily as an alternative to ritonavir in both treatment-naïve and treatment-experienced patients. As such, it is available as 150-mg tablets, but it is also available in fixed-dose combinations with atazanavir (Evotaz), with darunavir (Prezcobix, Rezolsta), with emtricitabine, TDF, elvitegravir (Stribild), and emtricitabine, TAF, elvitegravir (Genvoya).

Pharmacology

Cobicistat reaches its C_{max} of 0.99 μg/ml within 3.5 h postdose. There are no specific data for cobicistat with regard to food, and recommendations are based on the concomitant

antiretrovirals. When administered with atazanavir and darunavir, doses should be administered with food. Cobicistat is >97% protein bound. It is metabolized by CYP3A and CYP2D6 (minor pathway) and eliminated via the liver with a $t_{1/2}$ of ∽3 to 4 hours. No significant pharmacokinetic changes are noted in patients with mild to moderate hepatic impairment or in those with severe renal impairment. When combined with atazanavir in HIV-infected subjects, it shows comparable pharmacodynamics to ritonavir-boosted atazanavir and is noninferior in rates of virologic suppression (326, 344). Similarly, cobicistat-boosted darunavir results in similar plasma concentrations and adverse events as ritonavir-boosted darunavir in HIV uninfected healthy volunteers.

Adverse Effects

The most significant adverse effects reported in clinical trials combined with atazanavir were jaundice (6%) and rash (5%). Cobicistat also inhibits tubular secretion of creatinine without affecting glomerular filtration function. Thus a rise in serum creatinine may be seen that is not reflective of true renal toxicity. The mean decrease in estimated (but not actual) glomerular filtration rate in clinical trials has been 15 ml/min. No appreciable impact on lipid profiles has been noted.

Drug Interactions

Cobicistat has potent CYP3A inhibitory activity with 95% inhibition at a dose of 200 mg, similar to 96% with a ritonavir dose of 100 mg (255). Compared to ritonavir, cobicistat is a weaker inhibitor of CYP2D6 and demonstrates no inhibitory activity against other CYP450 isoenzymes. Cobicistat also inhibits P-glycoprotein, which further enhances concomitant antiretroviral absorption. Coadministration of cobicistat with the following combinations is not currently recommended due to concerns for low concentrations and virologic failure: 1) darunavir with efavirenz, nevirapine, or etravirine, 2) atazanavir with etravirine, 3) atazanavir with efavirenz in treatment-experienced patients, 4) darunavir twice daily, and 5) fosamprenavir, or saquinavir, or tipranavir. Cobicistat should also not be used with other ritonavir-boosted regimens. Cases of acute renal failure and Fanconi syndrome have been reported with concomitant use of cobicistat and TDF.

Resistance

Resistance to other antiretrovirals can develop during treatment with cobicistat-boosted regimens.

Clinical applications

Cobicistat has no direct antiretroviral activity but is used as a pharmacokinetic enhancer for protease inhibitors and the integrase inhibitor, elvitegravir. It is currently available in coformulations to boost elvitegravir, darunavir, and atazanavir. Rates of virologic suppression and adverse events during treatment with darunavir-cobicistat in HIV-infected individuals are similar to historical data from treatment with darunavir-ritonavir (345).

MATURATION INHIBITORS

Agents targeting later stages of the life cycle of the virus, such as BMS-955176, represent a new class of antiretroviral drugs, the maturation inhibitors (Table 2). This second-generation agent disrupts a late step in the processing of HIV-1 Gag that inhibits the last protease cleavage event between capsid protein p24 and spacer peptide 1 in Gag resulting in production of noninfectious virions with abnormal capsid morphology. As a second-generation agent, BMS-955176 binds reversibly but with greater affinity to HIV-1 Gag than its predecessor (bevirimat) and retains activity in the presence of Gag polymorphisms associated with reduced bevirimat susceptibility (346). Mutations for resistance to the drug have been found at the p25-to-p24 cleavage site. The drug is administered once daily and has generally been well tolerated in clinical studies in combination with boosted and unboosted atazanavir.

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Antiherpesvirus Agents

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ANTIHERPESVIRUS AGENTS

The first effective agents against herpesviruses were nucleoside analogs, such as idoxuridine, vidarabine, and trifluridine. Their use was eventually supplanted by the highly successful drug, acyclovir, which had significantly less toxicity. In early studies, intravenous acyclovir was shown to be superior to vidarabine for treating herpes simplex virus (HSV) encephalitis in healthy hosts and varicella zoster virus (VZV) infections in immunosuppressed subjects and to be equivalent to vidarabine in treating neonatal HSV infections.

Acyclovir

Acyclovir, 9-(2-hydroxyethoxy)methyl]guanine (Zovirax), (Fig. 1; Tables 1 and 2) represents a landmark in the history of antiviral drug development. The anti-HSV activity of acyclovir demonstrated that analogs of guanosine were active and that acyclic side chains could substitute for the ribose moiety, conferring specificity by the selective uptake and activation of acyclovir in HSV-infected cells.

Spectrum of Activity

Acyclovir's greatest, and clinically most important, activity is against HSV type 1 (HSV-1), HSV-2, and VZV. The inhibitory concentrations for susceptible isolates of HSV-1 average 0.04 μg/ml. HSV-2 is approximately 2-fold less susceptible, and VZV is approximately 8-fold less susceptible, than HSV-1. Acyclovir also has lower activity against Epstein-Barr virus (EBV) (1). Cytomegalovirus (CMV) susceptibilities are quite variable, and the range of susceptibilities is beyond achievable acyclovir levels for treatment (1). In addition, acyclovir has low efficacy at preventing CMV infection (2). Human herpesvirus (HHV)-6 is only modestly susceptible to acyclovir (3). HHV-8 is not susceptible to acyclovir (4, 5).

Mechanism of Action

Acyclovir is a poor substrate for cellular enzymes. Intracellular phosphorylation of acyclovir monophosphate is mediated by HSV or VZV thymidine kinase. In HHV-8- and EBV-infected cells, acyclovir is monophosphorylated by the viral protein kinases (6, 7). The di- and triphosphorylation of acyclovir occurs by cellular kinases to generate the active form of the drug, acyclovir triphosphate. Selectivity for HSV is conferred by the high affinity for acyclovir by HSV thymidine kinase and DNA polymerase, in contrast to the low affinity exhibited by host cell enzymes (8). Acyclovir triphosphate is both a competitive inhibitor of the viral DNA polymerase and a chain terminator. Because acyclovir lacks the 3′-hydroxy group necessary to form 3′-5′ phosphodiester bonds, incorporation of acyclovir triphosphate into the growing viral DNA chain leads to chain termination. Cellular DNA polymerases are much less susceptible to inhibition by acyclovir triphosphate (10- to 30-fold), which is another component of the selectivity of acyclovir.

Adverse Effects

The widespread use of acyclovir in both healthy and immunocompromised hosts for more than 30 years demonstrates that acyclovir is remarkably well tolerated. Following intravenous administration, local reactions at the injection site have been reported, and headache and nausea may occur. Neurotoxicity, albeit relatively rare, may manifest as tremors, myoclonus, confusion, lethargy, agitation, and hallucinations, as well as dysarthria, ataxia, hemiparesthesias, and seizures. Symptoms of neurotoxicity usually appear within the first 24 to 72 hours of administration and are more likely to occur when levels in plasma are elevated, as with intravenous administration or in the setting of renal insufficiency (9). Acyclovir is relatively insoluble in urine, with a maximum solubility of 2.5 mg/ml at physiological pH. As a result of the low urine solubility, acyclovir crystallization may occur in kidney tubules, especially in the setting of elevated plasma acyclovir levels, rapid intravenous bolus administration, and dehydration (10). Because of acyclovir's low isoelectric point (pI), the drug is less soluble at acid pH; therefore lowering the pH of the urine reduces the risk of crystallization. Prevention of acyclovir crystal deposition can be accomplished by volume repletion prior to drug administration and avoidance of rapid infusions. Acute tubular toxicity leading to renal failure has also been reported with acyclovir, especially for patients with underlying renal disease or receiving concomitant nephrotoxic drugs (11). Topical acyclovir is usually well tolerated, but there have been reports of local burning, stinging, and erythema (10).

In vitro, acyclovir is neither immunosuppressive nor toxic to bone marrow precursor cells. Although mutagenic in some preclinical assays, acyclovir at therapeutic doses lacks carcinogenicity and teratogenicity in animal studies. A large Danish cohort study and pregnancy registries found no

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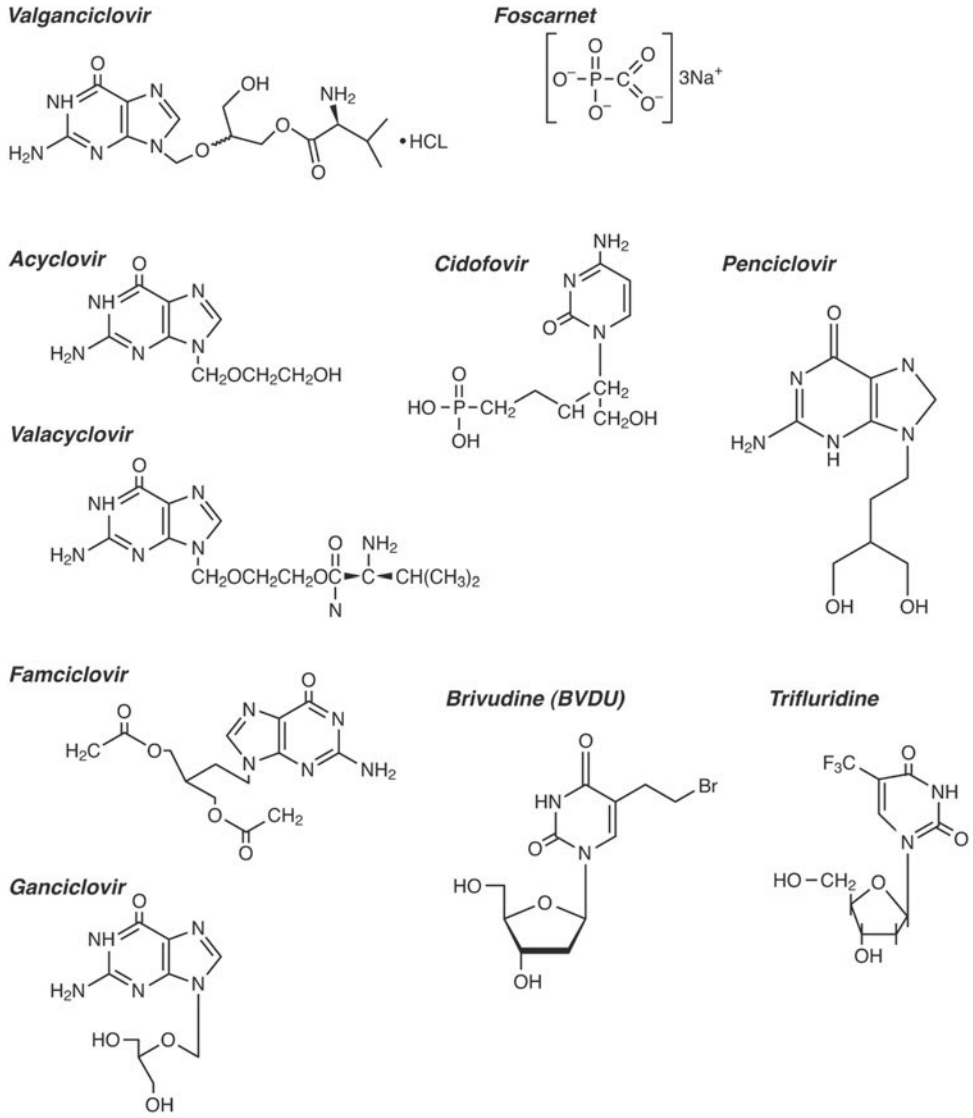

FIGURE 1 Chemical structures of antiherpesvirus agents. (Reprinted with permission from reference [230]).

association between acyclovir exposure and major birth defects (12, 13). In healthy subjects receiving chronic prophylaxis for genital HSV infection for over a decade, no chronic toxicities, including abnormalities of spermatogenesis, have been reported. HSV-specific immune responses can be diminished by acyclovir treatment of primary infection, which may be related to diminution of antigen expression (14).

Pharmacokinetics

Oral, intravenous, and topical preparations of acyclovir are available. Ophthalmic formulations are available outside the United States. The maximum plasma concentrations (C_{max}) average approximately 10 μg/ml after an intravenous infusion of 5 mg/kg of body weight and 0.6 μg/ml following a 200-mg oral dose; the levels in plasma proportionately increase with increasing doses (15). Concentrations achieved in the cerebrospinal fluid are approximately 50% of plasma values (15). Oral bioavailability is 15 to 30% and may be lower, on average, in immunocompromised hosts (15). Minimal, if any, drug is absorbed after topical administration. Acyclovir-elimination half-life ($t_{1/2}$) in plasma averages 3 hours in subjects with normal renal function. The initial intracellular $t_{1/2}$ of acyclovir triphosphate is 1.2 hours and reaches a plateau after 6 hours (16). The primary mode of excretion is renal and occurs through both glomerular filtration and tubular secretion. Approximately 85% of administered drug is excreted unchanged in the urine; the remainder is metabolized to 9-carboxymethoxymethylguanine before excretion. Dose adjustments for renal insufficiency, dialysis, and continuous renal replacement therapy are necessary. Where available, therapeutic drug monitoring may be helpful in guiding optimal therapy for patients in whom achieving therapeutic drug levels is especially critical (e.g., neonatal herpesvirus infection).

Drug Interactions

Few potentially important drug interactions have been noted. Caution should be exercised when other potentially nephrotoxic or neurotoxic agents are being used concur-

TABLE 1 Antiherpesvirus agents: approved and potential indications and dosing regimens

Agent	Formulation	Indication	Standard adult dosing[a]
Antiherpesvirus agents			
Acyclovir	Oral	Immunocompetent host	400 mg p.o. t.i.d. for 7–10 days *or* 200 mg p.o. 5×/day for 7–10 days or apply topically 6×/day for 7 days. For more severe cases (e.g., accompanied by aseptic meningitis, etc.), 5–10 mg/kg i.v. q8h initially, followed by p.o. to complete at least 10 days of total therapy
	Intravenous	Genital HSV infection	
	Topical	Primary	
		Recurrent	400 mg p.o. t.i.d. for 5 days *or* 800 mg p.o. b.i.d. for 5 days *or* 800 mg p.o. t.i.d. for 2 days initiated at earliest sign or symptom of recurrence
		Suppression	400 mg p.o. b.i.d.
		Herpes labialis	Apply 5×/day for 4 days
			400 mg p.o. 5×/day for 5 days
		Herpes simplex encephalitis or other invasive syndromes	10 mg/kg i.v. q8h for 14–21 days for encephalitis and 7–10 days for meningitis
		Varicella, chickenpox	800 mg p.o. 5×/day for 5 days *or* 10–15 mg/kg i.v. q8h for 10–14 days if invasive (e.g., encephalomyelitis)
		Herpes zoster, shingles	800 mg p.o. 5×/day for 7–10 days
		Immunosuppressed host	400 mg p.o. 5×/day for 7–10 days *or* 5 mg/kg i.v. q8h in more severe cases for 7 days
		Mucocutaneous HSV	
		Herpes labialis	Apply 6×/day for 7 days
		Suppression	400–800 mg p.o. b.i.d. or t.i.d.
		HSV encephalitis	10 mg/kg i.v. q8h for 14–21 days and 7–10 days for meningitis
		Herpes zoster, shingles	10 mg/kg i.v. q8h for 7 days
		Varicella, chickenpox	800 mg p.o. 5×/day for 7 days *or* 10–15 mg/kg i.v. q8h for 10–14 days if invasive (e.g., encephalomyelitis)
Valacyclovir	Oral	Immunocompetent host	1 g p.o. b.i.d. for 7–10 days
		Genital HSV infection	500 mg p.o. b.i.d. for 3 days *or* 1 g p.o. daily for 5 days
		Primary	1 g p.o. daily or 500 mg p.o. daily
		Recurrent	2 g p.o. b.i.d. for 1 day
		Suppression	1 g p.o. t.i.d. for 7 days
		Herpes labialis	
		Herpes zoster, shingles	
		HIV-infected host	1 g p.o. b.i.d. for 5–10 days
		Genital HSV	500 mg p.o. b.i.d.
		Recurrent	
		Suppression	
Famciclovir	Oral	Immunocompetent host	250 mg p.o. t.i.d. for 7–10 days
		Genital HSV infection	1 g p.o. b.i.d. for 1 day or 125mg p.o. b.i.d. for 5 days or 500mg once, followed by 250mg p.o. b.i.d. for 2 days
		Primary	
		Recurrent	250 mg p.o. b.i.d.
		Suppression	1,500 mg p.o. in one dose, initiated at earliest sign
		Herpes labialis	500 mg p.o. t.i.d. for 7 days
		Herpes zoster, shingles	
Penciclovir	Topical	Herpes labialis	Apply q2h for 4 days
Brivudine	Oral	Herpes zoster, shingles	125 mg p.o. daily for 7 days in immunocompetent host
	Ophthalmic	Herpes simplex keratitis	Apply to conjunctiva 5×/day
Trifluridine	Ophthalmic	Herpes simplex keratitis	1 drop of 1% solution q2h (maximum, 9 drops/day) for 10 days
Ganciclovir	Oral	HIV-infected host	5 mg/kg i.v. q12h for 14–21 days for induction treatment, followed by maintenance therapy (5 mg/kg i.v. daily for 7 days/wk *or* 6 mg/kg i.v. daily for 5 days/wk *or* 1 g p.o. t.i.d. or 500 mg 6×/day)
	Intravenous	CMV retinitis	

(*Continued*)

TABLE 1 Antiherpesvirus agents: approved and potential indications and dosing regimens (*Continued*)

Agent	Formulation	Indication	Standard adult dosing[a]
		CMV, gastrointestinal	5 mg/kg i.v. q12h for 21–28 days (need for maintenance not proven, but advisable)
		Other immunosuppressed host CMV infection	5 mg/kg i.v. q12h for 14–21 days (maintenance therapy not generally necessary)
		Prophylaxis or preemptive therapy of CMV in transplant recipients	5 mg/kg i.v. q12h for 7–14 days and then 5 mg/kg i.v. daily 7 days/wk *or* 6 mg/kg i.v. daily 5 days/wk during "risk" period for transplant patients *or* 1 g p.o. t.i.d. in solid-organ transplant patients
		CMV pneumonia in bone marrow transplant recipients	5 mg/kg i.v. q12h for 10–14 days combined with i.v. immune (or CMV hyperimmune) globulin
Valganciclovir	Oral	HIV-infected host CMV retinitis	900 mg p.o. b.i.d. for 21 days for induction therapy, followed by 900 mg p.o. daily for maintenance
		CMV prophylaxis in high-risk kidney, heart, and kidney-pancreas transplant patients	900 mg p.o. daily
Foscarnet	Intravenous	HIV-infected host CMV retinitis	60 mg/kg i.v. q8h *or* 90 mg/kg i.v. q12h for 14–21 days for induction treatment, followed by maintenance therapy (90–120 mg/kg i.v. daily)
		CMV, gastrointestinal or CNS disease	60 mg/kg i.v. q8h *or* 90 mg/kg i.v. q12h for 14–21 days (need for maintenance therapy not proven, but advisable)
		Other immunosuppressed host CMV disease	60 mg/kg i.v. q8h *or* 90 mg/kg i.v. q12h for 14–21 days
		HIV- or non-HIV-infected immunosuppressed host Acyclovir-resistant HSV or VZV infection	40 mg/kg i.v. q8h *or* 60 mg/kg i.v. q12h for 14 days
		Invasive HHV-6 disease	60 mg/kg i.v. q8h *or* 90 mg/kg i.v. q12h for 14–21 days
Cidofovir	Intravenous	CMV retinitis	5 mg/kg i.v. every wk for 2 wks and then 5 mg/kg i.v. every other wk (combined with probenecid and hydration for nephroprotection)
Docosanol	Topical	Recurrent herpes labialis	Apply topically 5×/day

[a]Abbreviations: p.o., per as; i.v., intravenously; 5×, five times; q.a.m., every morning; q.p.m., every evening.

rently, but whether there is additive toxicity with specific agents is unclear. *In vitro*, mycophenolate mofetil potentiates the anti-HSV activity of acyclovir through depletion of dGTP pools, but the clinical significance of this observation is unclear (17).

Resistance

The descriptions of persistent or progressive clinical disease with acyclovir-resistant HSV or VZV isolates in immunocompromised hosts, particularly patients with human immunodeficiency virus (HIV) infection, helped to establish the clear-cut clinical significance of antiviral resistance (18, 19). In the era of potent antiretroviral therapy, the incidence of this complication in HIV-infected persons has dramatically declined. The prevalence of acyclovir-resistant HSV infections in immunocompromised patients has increased in recent years and ranges from 3.5 to 11% (20–22). Hematopoietic stem cell transplant (HSCT) recipients are at greatest risk of acyclovir-resistant HSV infections with prevalences up to 47% in allogeneic HSCT recipients (20). HSV resistance in immunocompetent individuals generally occurs at a very low prevalence of 0.3–0.7% (23–25). Immunocompetent individuals receiving long-term acyclovir as prophylaxis for recurrent herpetic keratitis are at increased risk for resistance due to the immune-privileged nature of the cornea allowing for rapid selection of resistant strains in the absence of immune surveillance (26–29).

The vast majority of HSV resistance is based on mutations in the viral thymidine kinase gene, UL23, with the remainder of resistance based in the DNA polymerase gene, UL30 (30). Thymidine kinase-based resistance may be the result of absent or low-level enzyme production or the elaboration of a thymidine kinase with altered substrate specificity. Thymidine kinase deficiency is more often due to production of a truncated protein than to production of a functional, but altered, enzyme. Acyclovir-resistant subpopulations exist among clinical isolates of HSV in the absence of drug exposure, but the clinical expression of resistance only occurs in the face of drug treatment (31, 32). Such isolates may be the result of the selection of a preexisting drug-resistant subpopulation or a new mutational event.

Although acyclovir-resistant HSV strains are less pathogenic in animal models 33–37, they can cause serious clinical disease in immunocompromised hosts. In such hosts, HSV resistance is usually manifested by chronic mucocutaneous ulceration, but invasive visceral and central nervous system (CNS) disease, which may be fatal, occurs with either HSV-1 or HSV-2 (38). Following resolution of mucocutaneous lesions due to resistant HSV, subsequent recurrences are usually acyclovir susceptible (in the absence of acyclovir

TABLE 2 Antiherpesvirus agents: pharmacokinetics, major toxicities, and drug interaction characteristics

Agent	Oral bioavailability (%)	Effect of food on AUC	$t_{1/2}$ (h) in adults	Major route of elimination	Adjustment for renal insufficiency	Major toxicities	Major drug interactions
Acyclovir	15–30	No	3	85% renally excreted unchanged, catabolite also renally excreted	Yes	Headache, nausea, nephrotoxicity, neurotoxicity	Possible additive effects with other nephrotoxic or neurotoxic agents
Valacyclovir	55 (of acyclovir)	No	3	Renal	Yes	Same as acyclovir; possible association with HUS/TTP[b] when used in immunocompromised persons at high dosages	Same as acyclovir; cimetidine and probenecid decrease the rate but not the degree of valacyclovir-to-acyclovir conversion
Famciclovir	77	No	2	Penciclovir catabolite is renally excreted	Yes	Headache, nausea	Probenecid, theophylline: increased plasma famciclovir levels Digoxin levels increased
Brivudine	30	No	16	Metabolized by liver to inactive metabolite	No	Nausea, headaches	Potentiates toxicity of 5-FU or other fluoropyrimidines
Trifluridine	NA[a]	NA	NA	Negligible systemic absorption and metabolism	No	Local eye irritation; mutagenic, carcinogenic, and teratogenic potential	None reported
Ganciclovir	5	Increases by 8–9%	3–4	Mainly renally excreted unchanged	Yes	Neutropenia, thrombocytopenia	Bone marrow-suppressive agents: increased risk of myelosuppression
Valganciclovir	61	Increases by 14–30%	3–4	Mainly renally excreted unchanged	Yes	Neutropenia, thrombocytopenia	Bone marrow-suppressive agents: increased risk of myelosuppression
Foscarnet	NA	NA	4.5–8.2	Renal	Yes	Nephrotoxicity, electrolyte disturbances, neurotoxicity, anemia, neutropenia	Nephrotoxic agents: increased risk of foscarnet nephrotoxic adverse effects Pentamidine: increased risk of hypocalcemia
Cidofovir	NA	NA	2.6	Renal	Yes	Nephrotoxicity, neutropenia, uveitis, rash, iritis (with intravitreal administration)	Probenecid: nephroprotective at cidofovir doses of >3 mg/kg
Docosanol	NA	NA	NA	NA	No	Rash, pruritus, dry skin, burning and stinging at application site	None reported

[a]NA, not applicable.
[b]HUS/TTP, hemolytic-uremic syndrome/thrombotic thrombocytopenic purpura.

exposure), demonstrating that HSV-1 strains sensitive and resistant to acyclovir can coexist in latently infected ganglia (39). Recurrent disease due to thymidine kinase-altered virus in an immunocompetent host has been reported. Mutants retaining low-level production of thymidine kinase are able to reactivate more readily than thymidine-kinase deficient mutants (40, 41). HSV acyclovir resistance testing, where available by select commercial laboratories, can assist in identifying drug-resistant strains in refractory HSV infection. Identified genotypic mutations need to be confirmed as conferring resistance using established databases or phenotypic methods (30).

Clinical isolates of VZV, resistant to acyclovir from immunocompromised patients, present as chronic skin lesions unresponsive to therapy and are associated with significant morbidity and mortality due to visceral dissemination (42–44). The prevalence of acyclovir-resistant cases in different immunocompromised populations has not been determined, although a recent report suggested that approximately 27% of hemato-oncological patients with persistent VZV infections have acyclovir resistance (45). Similar to HSV, the majority of acyclovir resistance in VZV is due to mutations in the viral thymidine kinase and, less frequently, to mutations in the viral DNA polymerase gene (46).

Thymidine kinase-deficient or -altered isolates of HSV or VZV resistant to acyclovir remain susceptible *in vitro* to foscarnet, cidofovir, and brivudine but are fully cross-resistant to ganciclovir and famciclovir. When resistance is due to a DNA polymerase mutation, cross-resistance to foscarnet may exist, but susceptibility to cidofovir and brivudine is usually maintained (47).

Clinical Applications

Acyclovir has been extensively studied for the prophylaxis and treatment of herpesvirus infections in both immunocompetent and immunocompromised hosts. For serious mucocutaneous, visceral, or CNS disease due to HSV or VZV, parenteral acyclovir is the agent of choice unless acyclovir resistance is suspected. For patients with normal renal function, a dosage of 5 mg/kg every 8 hours (q8h) is appropriate for mucocutaneous disease. A dosage of 10 mg/kg q8h should be used for VZV infections and for invasive HSV disease. For neonatal HSV disease, a dosage as high as 20 mg/kg q8h has been recommended. Acyclovir at 10 mg/kg q8h is indicated for VZV disease because of the generally lower susceptibility of VZV isolates.

Acyclovir does not appear to be beneficial for patients with acute infectious mononucleosis, despite the demonstration of a decrease in EBV shedding, or for patients with chronic fatigue syndrome. There are limited data suggesting that acyclovir in conjunction with corticosteroids may be effective in severe primary EBV infection in immunocompetent patients (48). Oral hairy leukoplakia regresses with acyclovir treatment. Acyclovir has been reported anecdotally to induce regression in some patients with polyclonal B-cell posttransplantation lymphoproliferative disorders (PTLD). Antiviral therapy is often used in conjunction with reduced immunosuppression and other therapies in PTLD in the absence of clear evidence of acyclovir efficacy. Most EBV-infected cells within PTLD lesions are transformed B cells that are not undergoing viral lytic replication or expressing viral thymidine kinase and therefore will not be inhibited by acyclovir or ganciclovir. Prophylactic use of acyclovir or ganciclovir after transplantation may reduce the risk of PTLD, especially during the first posttransplantation year (49).

In healthy hosts, the intravenous and oral forms of acyclovir decrease the period of viral shedding and speed healing in patients with primary genital HSV infection (50). Of note, therapy for primary infections with intravenous acyclovir does not prevent the establishment of latency. Topical acyclovir ointment demonstrates some efficacy for primary genital HSV infection (51), but it is inferior to systemic therapy. For recurrent genital HSV infection, oral acyclovir for 5 days reduces the duration of pain and viral shedding. Topical acyclovir is clinically ineffective for recurrent genital HSV infection. Short-course acyclovir therapy, consisting of 2 days of 800 mg three times daily (t.i.d.), started within 12 hours of symptom onset, decreases time to healing and episode duration by 2 days compared to placebo (52). For recurrent orolabial HSV, a 5% acyclovir cream preparation has been approved by FDA (53), and oral acyclovir at 400 mg five times per day has been demonstrated to be effective.

Oral acyclovir is also beneficial for the treatment of herpes zoster in adults and varicella in children. The acute pain of herpes zoster is consistently diminished by acyclovir. A recent meta-analysis found that acyclovir was ineffective in preventing postherpetic neuralgia (PHN) (54). There is currently insufficient data to determine if valacyclovir and famciclovir are effective in preventing PHN (54). Oral acyclovir alone, or in combination with prednisolone, does not improve the likelihood of complete recovery from Bell's palsy beyond the benefit of prednisolone alone (55).

Acyclovir is also effective for prophylaxis of HSV infections in immunosuppressed patients undergoing solid-organ or bone marrow transplantation and in patients receiving cancer chemotherapy (56). Although therapeutically ineffective for established CMV infections, acyclovir does have efficacy for the prophylaxis of CMV infection in renal, liver, and bone marrow transplant recipients (57); however, this role has been largely supplanted by valganciclovir. Antiviral medications used to prevent CMV replication during preventative or treatment strategies (e.g., ganciclovir, valganciclovir, acyclovir, valacyclovir) also prevent HSV replication.

In healthy hosts, oral acyclovir is very effective for the suppression of recurrent genital herpes, reducing recurrences by approximately 90% (58, 59). Safety, efficacy, and lack of emergence of resistance for periods up to 10 years have been demonstrated (60). Subclinical shedding of HSV-2 from the genital tract still occurs during antiviral therapy and may account for ongoing transmission during suppressive antiviral therapy (61). Prevention of herpetic whitlow and recurrent erythema multiforme associated with recurrent HSV infection is also possible. Short-term prophylaxis of recurrent oral HSV infection can be effective when initiated prior to a known stimulus for reactivation, such as occurs with exposure to UV light (e.g., in skiers).

The use of intravenous acyclovir for the treatment of CNS infections and neonatal herpes is discussed in Chapter 20.

Valacyclovir

Valacyclovir (2-[2-amino-1,6-dihydro-6-oxo-9H-purin-9-yl-methoxy]ethyl-L-valinate hydrochloride, Valtrex) (Fig. 1; Tables 1 and 2) is the L-valyl ester of acyclovir. It was designed to enhance the oral bioavailability of the parent compound and is one of a series of such compounds that has been investigated. Valacyclovir is hydrolyzed to acyclovir by first-pass metabolism in the liver, prior to anabolic phosphorylation in the infected cell, thus avoiding toxicity. The antiviral spectrum of activity of valacyclovir is identical to that of acyclovir.

Mechanism of Action and Pharmacokinetics

The L-valyl esterification of acyclovir increases the bioavailability of acyclovir and does not alter the mechanism of action of the drug. Absorption of valacyclovir in the gastrointestinal tract is facilitated by a stereospecific transporter system; first-pass intestinal and hepatic hydrolysis by valacyclovir hydrolase yields complete conversion of valacyclovir to acyclovir, resulting in a 3- to 5-fold increase in acyclovir bioavailability. Once the drug is converted to acyclovir, the elimination $t_{1/2}$, excretion, and metabolism are the same as those noted for acyclovir. In healthy volunteers, given doses of 500 and 1,000 mg of valacyclovir, acyclovir C_{max} is reached in 1.5 to 1.75 hours and averages 3.3 and 5.7 μg/ml, respectively (62). Pharmacokinetic parameters in patients with advanced HIV infection are similar to those in healthy volunteers. Valacyclovir at 1,000 mg t.i.d achieves concentrations in the vitreous within the inhibitory ranges of HSV-1, HSV-2, and VZV (63). This dose regimen also provides inhibitory acyclovir levels in the cerebrospinal fluid of patients with HSV encephalitis and may have utility in resource-poor settings where intravenous acyclovir is unavailable but use of valacyclovir for this indication is not recommended (64).

Drug Interactions and Adverse Effects

The drug interaction and side effect profiles are similar to those of acyclovir. In patients with advanced HIV infection, gastrointestinal complaints and neutropenia were seen in 31 and 19% of subjects, respectively (65). Cases resembling thrombotic microangiopathy have been reported for subjects with advanced HIV infection and for recipients of allogeneic bone marrow transplants treated with high dosages of valacyclovir (8,000 mg/day) for prolonged periods for CMV prophylaxis (66), but such events have not been reported for immunocompetent or HIV-infected subjects receiving valacyclovir for suppression of genital herpes. Although a causal association with valacyclovir has not been elucidated, the implication is that the occurrence of thrombotic microangiopathy is restricted to severely immunocompromised individuals receiving high dosages of valacyclovir or sometimes other drugs. Safety data for acyclovir in pregnancy may be extrapolated to valacyclovir (12).

Resistance

Acyclovir-resistant HSV or VZV may emerge during valacyclovir therapy, and the mechanisms are similar to those reported for acyclovir.

Clinical Applications

Valacyclovir has demonstrated efficacy in the treatment of herpes zoster and genital HSV infections. Valacyclovir, at 1,000 mg twice a day (b.i.d.), was found to be comparable to acyclovir at 200 mg five times daily in terms of efficacy and safety for the treatment of initial genital herpes infection (67). Valacyclovir at 1,000 or 500 mg b.i.d. for 5 days has an efficacy comparable to that of acyclovir at 200 mg five times daily for 5 days in immunocompetent individuals with recurrent genital herpes (68). Short-course therapy with valacyclovir at 500 mg b.i.d. for 3 days was equivalent to the 5-day course of valacyclovir (69). For suppressive therapy for recurrent genital herpes, once-daily dosing with 500 or 1,000 mg valacyclovir appears to be as effective as acyclovir at 400 mg b.i.d. (59, 70). Valacyclovir, 2,000 mg b.i.d. for 1 day, is approved for the treatment of recurrent herpes labialis. Valacyclovir at 1,000 mg t.i.d. is an effective therapy for herpes zoster (71) and may be superior to acyclovir in reducing the risk of acute pain with herpes zoster (72). Valacyclovir alone does not prevent PHN; however, valacyclovir in combination with gabapentin, commenced during an episode of acute herpes zoster, has been shown to reduce rates of PHN (73). Oral valacyclovir at a dosage of 2,000 mg four times daily is effective in the prophylaxis of CMV (and HSV) infections in renal- and stem-cell-transplant recipients (74). In HIV-infected persons, valacyclovir appeared to be superior to acyclovir in preventing CMV disease in one report, but the study was terminated early because of a higher mortality rate in the valacyclovir arm, a finding that is unexplained (75). Valganciclovir has largely replaced valacyclovir for prophylaxis of CMV disease.

Valacyclovir at a dosage of 1,000 mg t.i.d. is an option for systemic oral antiviral treatment of acute retinal necrosis due to herpesviruses (76) and is preferred over oral acyclovir, as a step-down therapy after intravenous acyclovir, due to its superior penetration into the vitreous fluid (63).

Famciclovir and Penciclovir

Famciclovir, 9-(4-hydroxy-3-hydroxymethylbut-1-yl)guanine (Famvir) (Fig. 1; Tables 1 and 2), is the diacetyl 6-deoxy prodrug ester of penciclovir, 9-(4-hydroxy-3-hydroxy-3-hydroxymethylbut-1-yl)guanine, an acyclic nucleoside analog of guanosine.

Spectrum of Activity

Famciclovir is a prodrug lacking intrinsic antiviral activity and must be converted to penciclovir triphosphate, which is its active form. HSV-1, HSV-2, and VZV are susceptible to penciclovir; the average inhibitory concentrations are 0.4, 1.5, and 5.0 μg/ml in MRC-5 cells, respectively (77). EBV and hepatitis B virus (HBV) are also susceptible *in vitro*, but HHV-6 and HHV-8 are not susceptible to penciclovir (4). Famciclovir also shows antiviral activity in animal models of hepadnavirus infection but is not a clinically useful anti-HBV agent.

Mechanism of Action

Penciclovir is selectively monophosphorylated by the HSV or VZV thymidine kinase; formation of penciclovir di- and triphosphate is catalyzed by cellular kinases. Penciclovir triphosphate competitively inhibits the viral DNA polymerase and can also be incorporated into the growing viral DNA chain. Because of the presence of the hydroxyl group on the acyclic side chain, viral DNA chain extension may occur to a limited extent after incorporation (77). This aspect of its mechanism of action contrasts with that of acyclovir triphosphate, which is an obligate chain terminator. Penciclovir triphosphate is a less potent inhibitor of HSV DNA polymerase than acyclovir triphosphate, but this lower inhibition is balanced by much higher intracellular levels of penciclovir triphosphate and by its much longer intracellular $t_{1/2}$. The intracellular $t_{1/2}$ of penciclovir is 10 to 20 hours in HSV-infected cells and 9 hours in VZV-infected cells (77).

Pharmacokinetics

Famciclovir is rapidly converted to penciclovir by deacetylation and oxidation in the liver. Famciclovir undergoes extensive first-pass metabolism, with aldehyde oxidase probably responsible for the oxidation at the 6 position. Following an oral dose of 500 mg of famciclovir, a penciclovir C_{max} of 3.3 μg/ml is achieved within 1 hour (78). Sixty to 70% of the drug is excreted as penciclovir in the urine by both glomerular filtration and tubular secretion. The remainder is excreted in the feces or is eliminated as

6-deoxy-penciclovir and other minor metabolites in the urine or feces (78). In subjects with hepatic disease, the time-to-peak plasma drug level and the concentration achieved are decreased relative to those in healthy volunteers, but overall exposure is not reduced. Dosage adjustment is required in patients with renal insufficiency and in those receiving hemodialysis. There are currently no data to guide dosing in peritoneal dialysis and continuous renal replacement therapy. Oral famciclovir achieves intravitreous concentrations of penciclovir within the inhibitory ranges for HSV and VZV (79). Topical penciclovir preparations are available.

Adverse Effects

Famciclovir is generally very well tolerated. Headache and nausea are the most common side effects. Famciclovir and penciclovir have shown positive results in some preclinical carcinogenesis models, including rodent tumor induction and mutagenesis assays, although not in tests of teratogenicity. Famciclovir use in pregnancy has not been associated with major birth defects, although the data are more limited than for acyclovir (12).

Drug Interactions

Probenecid, or other drugs significantly eliminated by active renal tubular secretion, would predictably increase plasma penciclovir concentrations because of the inhibition of renal tubular secretion of the drug, but formal interaction studies have not been performed. Theophylline can increase penciclovir levels moderately, but dose adjustments are not necessary. Allopurinol has no effect on penciclovir pharmacokinetics. Penciclovir, on the other hand, can increase digoxin levels modestly (mean increase, 19%) but does not alter the digoxin area under the concentration-time curve (AUC). Drugs which are also metabolized by hepatic aldehyde oxidase (80) have the potential to interact with famciclovir, although no reports documenting clinical interactions have been identified (81).

Resistance

The prevalence of penciclovir-resistant HSV isolates among subjects participating in clinical trials involving penciclovir (topical and intravenous) or famciclovir were 0.2 to 0.3% and 2.1% in immunocompetent and immunocompromised subjects, respectively (82, 83). Most HSV and VZV isolates, resistant to acyclovir on the basis of thymidine kinase deficiency, are cross-resistant to famciclovir and penciclovir. Interestingly, mutations associated with penciclovir resistance in the thymidine kinase gene are distributed throughout the gene, in contrast to those associated with acyclovir, which are preferentially localized to the homopolymeric hot spots (82). Some acyclovir-resistant isolates with altered thymidine-kinase substrate specificity may still retain their ability to phosphorylate penciclovir and remain susceptible.

Clinical Applications

Famciclovir is approved in the United States for the treatment of genital herpes, herpes labialis, and herpes zoster. For recurrent genital herpes, famciclovir at 125 mg was similar to acyclovir at 200 mg in 5-day treatment courses (84). Short courses of famciclovir are approved for treatment of recurrent genital herpes (1,000 mg b.i.d. for 1 day) and herpes labialis (1,500-mg single dose) (85, 86). Topical 1% penciclovir cream has moderate efficacy in treatment of orolabial herpes, decreasing the time to healing, in comparison to placebo, by a median of 1 day (87). In the treatment of herpes zoster, famciclovir at 500 mg t.i.d. decreases the time to lesion healing and reduces pain (88). In a controlled trial comparing famciclovir dosages of 250, 500, and 750 mg t.i.d. to acyclovir at 800 mg five times per day in immunocompetent adults, acute measures of healing and pain were equivalent (89). Famciclovir at 500 mg t.i.d. has efficacy and tolerability similar to that of acyclovir at 800 mg five times daily in immunocompetent persons with ophthalmic zoster (90) and similar to that of valacyclovir at 1,000 mg t.i.d. in immunocompetent persons over 50 years of age with herpes zoster (91). Similar to acyclovir, famciclovir does not prevent PHN (54). Famciclovir at a dosage of 500 mg t.i.d. is an option for systemic oral antiviral treatment of acute retinal necrosis due to herpesviruses (76) as a step-down therapy from intravenous acyclovir (63).

Brivudine

Brivudine, (E)-5-(2-bromovinyl)-2′-deoxyuridine (Zostex, Zerpex, Brivirac, Helpin), is a selective nucleoside analog (Fig. 1; Tables 1 and 2) utilized for treatment of herpes keratitis and herpes zoster in immunocompetent patients in Europe.

Spectrum of Activity

Brivudine is a potent and selective inhibitor of HSV-1 and VZV replication. EBV is also sensitive to brivudine, but CMV and HSV-2 are relatively resistant to its antiviral action (92). *In vitro*, the 50% effective concentration (EC_{50}) of brivudine is 0.0024 μg/ml for VZV, compared to 4.64 μg/ml for acyclovir (93).

Mechanism of Action and Pharmacokinetics

Brivudine activation depends upon a specific phosphorylation by the HSV-1 or VZV thymidine kinase that converts brivudine to its 5′-monophosphate and 5′-diphosphate. HSV-2-encoded thymidine kinase is unable to phosphorylate brivudine 5′-monophosphate to 5′-diphosphate (94). After further phosphorylation by cellular kinases, brivudine 5′-triphosphate interacts with viral DNA polymerase either as a competitive inhibitor or as an alternative substrate allowing incorporation into the growing DNA chain. Incorporation may affect both the stability and function of viral DNA during the replication and transcription processes (95).

Brivudine has low bioavailability (30%) and is highly protein bound (>95%) to plasma protein. The C_{max} at steady state (1.7 μg/ml) is achieved 1 hour after administration of once-daily oral brivudine at 125 mg and is 1,700-fold greater than the *in vitro* 50%-inhibitory concentration (IC_{50}) for VZV. Brivudine is rapidly metabolized into inactive compounds, BVU [(E)-5(2-bromovinyl)uracil] and 2-deoxyribose-1-phosphate, by thymidine phosphorylase in the liver. However, BVU can be reconverted *in vitro* and *in vivo* to brivudine through a pentosyl transfer reaction, which restores the antiviral potential of the compound (96). Renal elimination predominates with 65% of the dose eliminated in the urine as metabolites; the plasma-terminal elimination $t_{1/2}$ is approximately 16 hours. No dose adjustments are necessary in patients with kidney or liver disease.

Adverse Effects

Oral brivudine is generally well tolerated, and the incidences of potentially treatment-related adverse events are similar for brivudine (8%) and acyclovir (10%), with nausea being the most commonly reported adverse event (97).

Drug Interactions
The main metabolite of brivudine, BVU, irreversibly inhibits dihydropyrimidine dehydrogenase, the enzyme that regulates the metabolism of natural nucleosides like thymidine and other pyrimidine derivatives, including the fluoropyrimidine, 5-fluorouracil (5-FU). Coadministration of brivudine and 5-FU increases the systemic exposure to 5-FU and increases its toxicity. When a congener analog, bromovinyl arabinosyl uracil (BVaraU), was administered in Japan to patients receiving 5-FU, several deaths occurred. In healthy adults who received brivudine at 125 mg once daily for 7 days, dihydropyrimidine dehydrogenase activity was fully restored by 18 days after the final dose of brivudine. Therefore, coadministration of brivudine and fluoropyrimidine derivatives, or administration of these agents within 4 weeks of each other, is contraindicated (98).

Resistance
Brivudine resistance occurs with viral strains that are thymidine-kinase deficient. There are no reports of clinical isolates with brivudine resistance that developed during therapy. A broad variety of HSV-1 clones can be selected under a single round of high-dose brivudine, including acyclovir-susceptible, but brivudine-resistant, phenotypes. Mutations associated with brivudine resistance occur in the homopolymer stretches of Gs and Cs of the thymidine kinase gene (99). Serial passage of VZV strains under the pressure of acyclovir, brivudine, or BVaraU yields variants cross-resistant to all drugs that depend on the viral thymidine kinase for activation (100). In contrast, virus strains selected under pressure of penciclovir are resistant to acyclovir but not to brivudine.

Clinical Applications
Brivudine is available in Europe for topical treatment of herpetic keratitis as 0.1% eye drops and orally for treatment of VZV and HSV-1. The efficacy of brivudine at 125 mg orally once daily for 1 week for treatment of herpes zoster in immunocompetent subjects >50 years of age is generally comparable to that of acyclovir (97) or famciclovir (101). Incidence of postherpetic neuralgia was lower with brivudine than with acyclovir (33%, versus 44%, respectively) (97), but similar to that with famciclovir (21% vs. 20%, respectively) (101). Brivudine appears to be effective for treatment of herpes zoster in immunocompromised patients but at higher doses than for acyclovir, 7.5 mg/kg/day in divided doses (102) or 125 mg every 6 hours (103). Topical brivudine has also been utilized to treat herpetic keratitis that is clinically resistant to other antiviral agents (104).

Trifluridine
Trifluridine, 5-trifluoromethyl-2′-deoxyuridine (Viroptic), is a fluorinated thymidine analog (Fig. 1; Tables 1 and 2) that is approved in the United States for the treatment of herpetic keratitis.

Spectrum of Activity
Trifluridine has activity against the DNA viruses HSV-1 and -2, CMV, and vaccinia virus; there is inconsistent activity against adenovirus. Inhibitory concentrations for strains of HSV average 10 μg/ml, and, importantly, acyclovir-resistant strains of HSV remain susceptible to trifluridine (105).

Mechanism of Action and Pharmacokinetics
Trifluridine is anabolically phosphorylated by cellular kinases to the triphosphate form, which is a competitive inhibitor of the HSV DNA polymerase. Trifluridine is available only as a 1% ophthalmic solution in the United States. There is no significant systemic absorption.

Adverse Effects
The most frequent adverse reactions reported during clinical trials were mild, transient burning or stinging upon instillation (5%) and palpebral edema (3%). Other, less common adverse reactions were superficial punctate keratopathy, epithelial keratopathy, hypersensitivity reaction, stromal edema, keratitis sicca, hyperemia, and increased intraocular pressure. The potential for corneal epithelial keratopathy is lower with trifluridine than with topical idoxuridine. Trifluridine has shown carcinogenic, mutagenic, and, potentially, teratogenic activities in preclinical assays.

Drug Interactions
No adverse drug interactions have been reported with the simultaneous treatment of the eye with topical antibacterial, steroid, or atropine preparations.

Resistance
No reports of clinical isolates of HSV resistant to trifluridine have appeared, although there is one report of laboratory selection of trifluridine resistance (106).

Clinical Applications
The primary and approved indication for topical trifluridine is for primary keratojunctivitis and recurrent epithelial keratitis due to HSV types 1 and 2. Topical trifluridine and acyclovir are comparable in therapeutic effectiveness, and both appear more effective than idoxuridine or vidarabine (107). Topical trifluridine has also demonstrated clinical utility in the treatment of persistent cutaneous ulcers, due to acyclovir-resistant HSV infections in patients with AIDS, and can be considered when faced with HSV ulcers that are due to acyclovir- and foscarnet-resistant strains.

Ganciclovir
Ganciclovir, 9-(1,3-dihydroxy-propoxymethyl)guanine (Cytovene) (Fig. 1; Tables 1 and 2), is an acyclic nucleoside analog of guanosine that is structurally similar to acyclovir, differing only by the presence of a hydroxymethyl group at the 3′ position of the acyclic side chain. This difference confers markedly improved activity against CMV but also a different toxicity profile.

Spectrum of Activity
The clinical usefulness of ganciclovir derives from its improved anti-CMV activity; inhibitory concentrations are 0.1 to 1.6 μg/ml for susceptible clinical isolates in contrast to 2.2 to 25 μg/ml with acyclovir (108). Ganciclovir also has activity against HSV-1 and -2, VZV, and EBV, with *in vitro* inhibitory concentrations in the range of 1- to 3-, 1- to 2-, and 1- to 5 μg/ml, respectively (108). It is inhibitory for HHV-6 and HHV-8 (7). Activity against HBV has also been demonstrated but is not clinically useful.

Mechanism of Action
As with other nucleoside analog antiherpes viral agents, ganciclovir requires intracellular phosphorylation to transform to its active form. Monophosphorylation in HSV- and VZV-infected cells is mediated by the virus-encoded thymidine kinase that phosphorylates acyclovir (108). In CMV-infected cells, ganciclovir is monophosphorylated by a

phosphotransferase, which is the product of the viral UL97 gene. In HHV-6-infected cells, ganciclovir in monophosphorylated by the viral U69 kinase (109). In HHV-8 and EBV-infected cells, ganciclovir is monophosphorylated by viral protein kinases (6, 7). Ganciclovir monophosphate is converted to the di- and triphosphorylated derivatives by cellular kinases. Ganciclovir triphosphate is a competitive inhibitor of herpesviral DNA polymerases and is incorporated into the growing viral DNA chain, where it slows chain elongation (108). The intracellular $t_{1/2}$ of ganciclovir triphosphate in CMV-infected cells is >18 hours; the prolonged intracellular $t_{1/2}$ of ganciclovir triphosphate compared with that of acyclovir triphosphate may be partially responsible for the improved anti-CMV activity of ganciclovir.

Pharmacokinetics

Ganciclovir is available in intravenous and ophthalmic formulations and can be administered by direct intravitreal injection and through an implantable intraocular device (no longer available in the United States). Following an intravenous dose of 5 mg/kg, the C_{max} is 8 μg/ml; dose-independent kinetics have been observed at intravenous doses of ≤5 mg/kg (110). Intravitreal levels averaging 1.2 μg/ml have been reported for patients on induction doses of intravenous ganciclovir (111). Despite poor oral absorption, peak levels in plasma of 1 μg/ml can be achieved with a single oral dose of 1,000 mg taken with food. However, the bioavailability of oral valganciclovir, which results in plasma ganciclovir levels comparable to that seen with parenteral administration, has supplanted the use of oral ganciclovir, which is no longer available. CNS penetration occurs, with ratios of drug concentrations in cerebrospinal fluid (CSF) to those in plasma ranging from 0.24 to 0.70. Ganciclovir is mainly renally excreted in an almost unchanged form with an average elimination $t_{1/2}$ of 2 to 4 hours in subjects with normal renal function. Dosage adjustments are necessary in patients with renal impairment, receiving dialysis, or on continuous renal replacement therapy.

Adverse Effects

Hematologic toxicity is the most important adverse reaction noted following intravenous ganciclovir administration (112). Absolute neutrophil counts below 1,000/mm^3 have been noted in approximately 40% of patients with HIV infection and in 7% and 40% of heart and bone marrow transplant recipients, respectively. Thrombocytopenia (platelet counts of <50,000/mm^3) occurs with a reported frequency of 13% in HIV-infected subjects and in 8 to 57% of transplant recipients. Granulocyte and granulocyte-macrophage colony-stimulating factors are effective in reversing the neutropenia associated with ganciclovir and permit its continued use at efficacious doses (113).

Other adverse effects noted in patients receiving ganciclovir include headache, neurotoxic reactions, fever, rash, gastrointestinal complaints (including diarrhea) in subjects taking the oral capsule formulation, anemia, and abnormal liver enzymes (112).

Retinal detachment may occur during the course of treatment of CMV retinitis (114). Detachment results from scarring and retraction as the infection is controlled or as the disease progresses. Detachments are probably not due to a direct toxic effect of the drug and are also seen in untreated patients, especially newborns with congenital CMV infection. In patients treated locally with ganciclovir via an implanted device, local inflammatory reactions, scleral thinning, and retinal detachments have been described. Ganciclovir is mutagenic, carcinogenic, and teratogenic in preclinical assays and inhibits the growth of bone marrow progenitor cells *in vitro*.

Drug Interactions

In HIV-infected subjects, the use of zidovudine in combination with induction doses of ganciclovir results in a high incidence of neutropenia that often precludes their simultaneous use, unless granulocyte or granulocyte-macrophage colony-stimulating factor support is employed. Similarly, caution must be exercised when administering potential marrow-suppressive agents to any individual receiving ganciclovir.

Resistance

HSV and VZV isolates, that are resistant to acyclovir on the basis of thymidine kinase deficiency, exhibit cross-resistance to ganciclovir. HSV isolates, resistant to acyclovir on the basis of mutations in the viral DNA polymerase, may remain susceptible to ganciclovir. Given the relative infrequency compared to DNA polymerase mutants to thymidine kinase-deficient mutants, ganciclovir should not be used empirically in cases in which acyclovir resistance is suspected or proven, unless the resistance mechanism is known to be DNA polymerase. *In vitro* studies have shown the potential for HHV-6 to exhibit resistance to antivirals; however, few clinical cases have been reported (115–117).

Two basic mechanisms cause resistance to ganciclovir in CMV clinical isolates. The first, and more common, is due to point mutations in the UL97 phosphotransferase gene, which encodes a protein kinase responsible for the monophosphorylation of ganciclovir. Almost all mutations of the UL97 gene have been found within two clusters of codons, codons 460 to 520 and 590 to 607, spanning the proposed ATP-binding and substrate-recognition sites, respectively (118). Eight UL97 mutations (single amino acid substitutions M460V/I, H520Q, C592G, A594V, L595S, L595F/W or C603W) account for ganciclovir resistance in most cases (119, 120), conferring a 3- to 15-fold increase in the ganciclovir concentration required to reduce viral growth *in vitro* (119). The overall level of drug resistance increases as mutations accumulate (119). High-level ganciclovir-resistant isolates are associated with the presence of both UL97 and DNA polymerase mutations (121). Less frequent UL97 mutations in the codon range 590 to 607 confer varying levels of ganciclovir resistance. Isolates, resistant to ganciclovir on the basis of UL97 gene mutations, remain susceptible to foscarnet and cidofovir. The second, and less common, mechanism of ganciclovir resistance in clinical CMV isolates consists of mutations in the conserved subdomains of the UL54 DNA polymerase gene (118). In such instances, cross-resistance to cidofovir or foscarnet may be present. Mutations in UL54 are diverse and cluster in particular functional domains. Mutations in the exonuclease and thumb domain generally confer ganciclovir and foscarnet cross-resistance, possibly by overcoming the idling of the polymerase complex to permit chain extension beyond the misincorporated base (122). Mutations in both the UL97 and UL54 genes may coexist and lead to high-level ganciclovir resistance. Some UL97 or UL54 mutations confer low levels of ganciclovir resistance and may be amenable to ganciclovir dose escalation (up to 10 mg/kg b.i.d.) in the absence of severe disease.

Before the advent of the era of potent antiretroviral therapy, the degree of immunosuppression in individuals with AIDS, combined with the need to continue

maintenance therapy in subjects with retinitis, provided an appropriate milieu for viral resistance to emerge (123). In one series of CMV retinitis patients, 7.6% excreted resistant virus following 3 or more months of ganciclovir therapy (123). Infection with ganciclovir-resistant CMV has been associated with increased morbidity and mortality in solid organ transplant recipients (124–126). In this population, long durations of oral ganciclovir or valganciclovir prophylaxis have not been associated with an increase in ganciclovir-resistant CMV disease due to a lack of viral replication (126). An alternative option to the prevention of CMV disease after transplant, the preemptive approach, has a higher frequency of drug-resistance emergence (127–129). CMV seronegative recipients of CMV-seropositive donor organs (D+/R-) are at increased risk for CMV drug resistance, with resistance developing in 5 to 12% (126). Other groups at risk of ganciclovir resistance include haploidentical or allogeneic HSCT recipients (incidence of 15%) and infants receiving long-term ganciclovir or valganciclovir therapy for congenital CMV infection (129–131). CMV ganciclovir resistance should be suspected in transplant recipients who have received several weeks of full-dose ganciclovir therapy and show a persistent or increasing CMV viral load or disease progression. CMV resistance testing can assist in identifying drug-resistant strains in refractory CMV infection. Genotypic assays detect the presence of viral-sequence variations known to be associated with antiviral resistance and are commonly used in clinical practice (119). Newly identified genotypic mutations need to be confirmed as conferring resistance using phenotypic methods available in research laboratories (119). Subtherapeutic ganciclovir dosing can lead to incomplete inhibition of viral replication and to selection of drug-resistant strains (132). Therapeutic drug monitoring may provide dosage guidance but is not routine and has not been validated in prospective studies (118, 126).

Clinical Applications

Ganciclovir is useful for the treatment and prophylaxis of CMV infections in immunocompromised hosts. In HIV-infected patients with CMV retinitis, rates of initial response to intravenous ganciclovir are generally 75 to 85% (133). Induction courses of 5 mg/kg b.i.d. for 14 to 21 days must be followed by maintenance therapy for at least 3 to 6 months, until the CD4+ cell count has risen to $>100/mm^3$ on antiretroviral therapy (ART) and with the concurrence of an ophthalmologist; otherwise, predictable recurrence and progression of CMV retinitis will occur in the absence of immune restoration by potent antiretroviral therapy. Foscarnet is comparable to ganciclovir as primary therapy for CMV retinitis and was reported to confer a median 4-month survival advantage before the availability of potent antiretroviral therapy (134). Ganciclovir, delivered locally by ocular implants, is effective in controlling CMV retinitis in patients intolerant of systemic ganciclovir. The combination of ganciclovir implants and oral ganciclovir has been shown to provide both systemic and local drug activity with decreased progression of existing disease and decreasing incidence of new CMV disease (135). However, the use of implants has declined dramatically in the era of potent antiretroviral therapy, and they are no longer manufactured. The immune-response inflammatory syndrome can occur in patients with CMV retinitis who are treated with ART, so close monitoring for this complication is indicated.

Ganciclovir is effective for gastrointestinal disease due to CMV in HIV-infected subjects, although the need for maintenance therapy is still debated in the absence of antiretroviral therapy. CNS disease and CMV polyradiculopathy have more variable response rates. Combination regimens with foscarnet suggest that these two agents can be used safely when administered concurrently or in alternating regimens (114). In severe or immediately life-threatening circumstances, such as CMV encephalitis, combined initial treatment with ganciclovir and foscarnet is a consideration. *In vitro* data suggest that a combination of ganciclovir and foscarnet is synergistic against ganciclovir-susceptible or weakly resistant CMV isolates; however, there are insufficient clinical data to determine whether combination antiviral therapy is superior to monotherapy.

In other immunocompromised hosts, such as solid-organ transplant recipients, ganciclovir appears to be effective for treating invasive CMV disease, but responses in bone marrow transplant recipients are variable. Ganciclovir monotherapy is ineffective in treating CMV pneumonia in this population, and ganciclovir was not better than placebo for CMV gastrointestinal disease (136). Early studies showed improved outcomes with ganciclovir combined with immunoglobulin for the treatment of CMV pneumonia in HSCT recipients. However, a recent large retrospective study showed a modest improvement in outcomes over the past 25 years, largely attributed to antiviral treatment and changes in transplant practices rather than to immunoglobulin-based treatments (137).

Ganciclovir has been studied extensively for prevention of CMV disease in organ transplant recipients. Effective strategies include prophylaxis, targeted prophylaxis, and preemptive therapy. Prophylaxis refers to administration of ganciclovir to all patients prior to detection of CMV viremia during the highest-risk period, usually for a predetermined time after transplantation. Targeted prophylaxis refers to administration of drug during a defined period of increased immunosuppression, such as OKT3 or antilymphocyte globulin treatment for rejection, prior to detection of CMV viremia. Preemptive therapy refers to initiation of treatment following the detection of CMV viremia or a positive CMV antigen test in the absence of clinically evident CMV disease.

Ganciclovir and foscarnet are considered first line therapies for established HHV-6 disease in transplant recipients. Ganciclovir in combination with other therapies may be efficacious in multicentric Castleman's disease because of the presence of active HHV-8 replication in association with this disease (138). Intravitreal ganciclovir can be used for acute retinal necrosis due to herpesviruses in combination with systemic antivirals (76).

Topical ganciclovir is licensed for the treatment of herpes simplex keratoconjunctivitis.

Valganciclovir

Valganciclovir, L-valine, 2-[(2-amino-1,6-dihydro-6-oxo-9H-purin-9-yl)methoxy]-3-hydroxypropyl ester, monohydrochloride (Valcyte), is the L-valyl ester of ganciclovir and was designed, in a manner similar to that for valacyclovir, as a prodrug to enhance the oral bioavailability of the parent compound (Fig. 1; Tables 1 and 2). It has been approved for treatment of CMV retinitis in patients with HIV infection and for prevention of CMV disease in high-risk kidney, heart, and kidney-pancreas transplant patients.

Spectrum of Activity

Since valganciclovir is completely converted to ganciclovir, their spectra of activity are identical.

Pharmacokinetics

Valganciclovir is available in oral formulations. Valganciclovir is rapidly absorbed and hydrolyzed by intestinal and hepatic esterases to ganciclovir and exhibits an approximately 10-fold increase in oral bioavailability compared to oral ganciclovir (139). Ganciclovir levels are detected in serum within 15 minutes of valganciclovir administration. In healthy volunteers, given 900 mg of valganciclovir, C_{max} averaged 5.6 μg/ml. The C_{max} and the time to attain the peak were greater and shorter, respectively, than those achieved with oral ganciclovir. A dose of 900 mg of valganciclovir produces the same drug exposure as a 5-mg/kg intravenous dose of ganciclovir in liver transplant recipients (140). Dosage adjustments are necessary in patients with renal impairment. Limited data support the use of valganciclovir in patients receiving dialysis. For treatment of CMV infection in hemodialysis patients, a recent guideline recommends 200 mg of the oral powder formulation three times weekly for induction and 100 mg three times weekly for maintenance (141). For CMV prophylaxis, 450 mg two- or three times weekly has been shown to be safe and effective in patients receiving hemodialysis (142). For patients receiving continuous renal replacement therapy, 450 mg every 48 hours for been recommended for CMV prophylaxis based on data in two patients (143).

Adverse Effects

In the pharmacokinetic studies to date, headache, nausea, and diarrhea have been most frequently noted, with neutropenia also seen with longer-term dosing.

Drug Interactions

No formal drug interaction studies with valganciclovir have been reported, but the profile would be expected to be similar to that for ganciclovir.

Resistance

Ganciclovir-resistant CMV may emerge during valganciclovir therapy, and the mechanisms are similar to those reported for ganciclovir. Initial studies in solid-organ transplant patients receiving valganciclovir prophylaxis suggest that genotypic resistance is rare (144). However, among 225 CMV donor-seropositive (D+)- recipient-seronegative (R-) kidney, pancreas, liver, and heart transplant recipients, 9 of 65 (15%) patients who developed CMV disease had clinically suspected drug-resistant virus, and 4 had confirmed UL97 or UL54 mutations (145). Allograft loss and mortality occurred in two of four patients with proven drug-resistant CMV disease and in three of five patients with clinically suspected drug-resistant CMV disease. Among 607 lung transplant recipients (D+/R- and R+), receiving valganciclovir prophylaxis for ≥6 months, CMV infections were diagnosed in 28% (170/607), and UL97 resistance mutations were detected in 9.4% (16/170) (124).

Clinical Applications

In HIV-infected subjects, oral valganciclovir (900 mg b.i.d. for 3 weeks and then 900 mg once daily for 1 week) has been shown to be comparable to intravenous ganciclovir in the induction phase of treatment for CMV retinitis (146). At week 4, 86% of ganciclovir recipients and 88% of valganciclovir recipients showed no evidence of disease progression by fundoscopic photography, and the percentage of subjects with CMV viremia declined comparably in both groups. The approved dosage of valganciclovir is 900 mg orally b.i.d. for induction therapy of CMV disease and 900 mg once daily for maintenance and prophylaxis after transplantation, adjusted for renal function. Valganciclovir and intravenous ganciclovir are both recommended for the treatment of nonsevere CMV disease (147), when gastrointestinal disease does not impair adequate bioavailability of valganciclovir. Length of treatment is determined by the monitoring of weekly CMV viral loads and continuing treatment until one or two consecutive negative samples are obtained with a minimum treatment course of 2 weeks.

The benefit of valganciclovir therapy to prevent CMV disease after solid-organ transplantation is well established, but the best strategy (preemptive or prophylactic), treatment regimen (full-dose or low-dose valganciclovir), and duration have not yet been fully delineated (128). Prophylaxis is preferred for D+/R- heart or lung transplant recipients based on the data suggesting better graft survival and clinical outcomes (148). Recommended durations of prophylaxis for high-risk D+/R- recipients is 6 to 12 months for lung transplant recipients, more than 6 months for intestinal transplant recipients, 6 months for renal recipients, and 3 to 6 months for liver, heart, and pancreas transplant recipients (128). Variation in duration of prophylaxis is dependent on the degree of immunosuppression, including the use of antithymocyte antibodies. Recommended durations of prophylaxis for R+ recipients are ≥6 months for lung transplant recipients, 3 to 6 months for those receiving potent immunosuppression or intestinal transplant recipients, and 3 months for kidney, pancreas, liver, and heart transplant recipients (128). There are insufficient data to support the use of half the recommended dose of valganciclovir for prophylaxis (i.e., 450 mg daily in patients with normal renal function) although some centers have reported success (128). Furthermore, prolonged low ganciclovir levels may result in high rates of ganciclovir resistance, as reported with prolonged oral ganciclovir use (149). CMV preemptive therapy is more widely used than prophylaxis in HSCT recipients because of the bone marrow toxicities of ganciclovir and the equivalence of clinical outcomes (150).

There are conflicting reports on the benefit of preemptive anti-CMV therapy in HIV-infected patients with persistently low CD4+ T cells, and any benefit must be balanced with risk of drug toxicity and the low rate of CMV end-organ disease in the potent ART era (151–153).

Symptomatic congenital CMV disease involving the central nervous system is routinely treated with 6 weeks of intravenous ganciclovir or oral valganciclovir. A recent randomized controlled trial demonstrated the benefit of 6 months of valganciclovir, as compared to 6 weeks, on long-term audiologic and neurodevelopmental outcomes. See Chapter 23 (154).

Foscarnet

Foscarnet, trisodium phosphonoformate (Foscavir) (Fig. 1; Tables 1 and 2) is a pyrophosphate analog that differs from the other major antiherpes viral agents in that it is not a nucleoside or nucleotide analog.

Spectrum of Activity

Foscarnet has *in vitro* activity against HSV-1 and -2, VZV, CMV, EBV, HHV-6, HIV, and HBV. Inhibitory concentrations of foscarnet for viral DNA polymerases are 0.4 to 3.5 μM for HSV-1, 0.6 to 22 μM for HSV-2, 0.4 μM for VZV, 0.3 μM for CMV, 0.5 to 3.0 μM for EBV, and 0.1 to 0.5 μM for HIV. Inhibitory concentrations for viral replication vary considerably. For example, the IC_{50} of foscarnet for isolates

of HSV, CMV, and HIV are 10 to 130, 100 to 300, and 10 to 25 μM, respectively (155). The different concentrations needed for enzyme inhibition and inhibition of viral replication are due to inefficient transport of this highly charged molecule across cell membranes. Isolates of HSV, VZV, and CMV, resistant to acyclovir and ganciclovir on the basis of diminished ability to phosphorylate these agents, remain susceptible to foscarnet.

Mechanism of Action

Foscarnet differs from nucleoside-analog inhibitors in a number of important respects: foscarnet does not require intracellular metabolism for activation, is a noncompetitive inhibitor of viral DNA polymerases, and is not incorporated into the growing viral DNA chain. It blocks the pyrophosphate-binding site on the viral DNA polymerase, thereby interfering with pyrophosphate exchange from deoxynucleotide triphosphate moieties.

Pharmacokinetics

Foscarnet is available as an intravenous formulation and can be administered as a continuous or intermittent infusion. Following intravenous administration of doses of 60 and 90 mg/kg, the C_{max} is 509 and 766 μM, respectively (156). The initial elimination $t_{1/2}$ in plasma is 4.5 to 8.2 hours in subjects with normal renal function, but there is a more prolonged terminal elimination $t_{1/2}$ that may extend as long as 88 hours. This prolongation relates to the deposition of the drug in bone, which can account for 20% of an administered dose (156). Foscarnet is mainly eliminated by the renal route, by glomerular filtration and tubular secretion. The CSF-to-plasma ratio of foscarnet averages 0.66, but a wide interpatient variability exists (157). Dose adjustments for renal insufficiency are necessary. Foscarnet is not recommended for patients undergoing hemodialysis because dosage guidelines have not been established.

Adverse Effects

Foscarnet has a number of important potential side effects. The most frequent is nephrotoxicity, with elevations of creatinine noted in the majority of treated subjects. Nephropathy related to foscarnet is due to reversible tubular lesions caused by degeneration of tubular epithelial cells often associated with foscarnet crystals, tubulointerstitial lesions resulting from toxic tubular injury, and glomerular lesions with obstructing foscarnet crystals. Systemic foscarnet crystal precipitation with intravascular obstruction has been reported and can lead to multiorgan failure (158, 159). Saline hydration can reduce the risk of nephrotoxicity.

Metabolic abnormalities include hypo- and hypercalcemia, hypomagnesemia, hypokalemia, and hypo- and hyperphosphatemia. Direct complexing of ionized calcium by foscarnet in the plasma may be responsible for hypocalcemia, and inhibition of the tubular reabsorption of phosphate may be responsible for the hypophosphatemia. Decreases in ionized calcium may not be reflected by the total serum calcium, and thus close observation for manifestations of hypocalcemia, such as paresthesias and tetany, is important.

Neurologic toxicities include tremor, altered sensorium, and seizures; the last is potentiated by the tendency for foscarnet to cause hypocalcemia. Anemia and neutropenia occur in as many as 33 and 17% of patients, respectively. Other side effects include headache, nausea, vomiting, and genital ulcerations, the last probably the result of local toxicity from the high concentrations of foscarnet excreted in the urine. In some test systems, foscarnet has demonstrated mutagenic and teratogenic effects, and its safety in pregnancy has not been evaluated. Its poor solubility requires administration of large fluid volumes; slow infusion is needed to minimize the risks of seizures and other manifestations of hypocalcemia.

Drug Interactions

Foscarnet should be used with particular caution in patients receiving pentamidine, because hypocalcemia can be potentiated by their concomitant use. Zidovudine and foscarnet exhibit no pharmacokinetic interactions, but together they may increase the risk for anemia (160). Elimination of foscarnet may be impaired by medications that inhibit renal tubular secretion. Any potentially nephrotoxic agent should be used with caution in patients receiving foscarnet. Ganciclovir exhibits no pharmacokinetic interactions with foscarnet, but dose adjustments of both drugs are necessary if a decrease in creatinine clearance occurs.

Resistance

Since foscarnet does not require intracellular activation, resistance to this agent occurs exclusively at the level of the viral DNA polymerase. Clinical isolates of HSV, CMV, and VZV, resistant to foscarnet, have been described, and the *in vitro* susceptibility of HSV strains derived from HIV patients correlates with foscarnet treatment success or failure (24, 161–170). For resistant isolates of HSV, CMV, and VZV, there are 3- to 8-fold increases in inhibitory concentrations, and progressive CNS disease has been associated with foscarnet-resistant HSV strains (171). Foscarnet-resistant HSV and CMV strains may remain susceptible to acyclovir and ganciclovir, respectively, although dually resistant clinical isolates have been described (172–174). For such isolates, cidofovir remains a therapeutic option. Resistance of HIV to foscarnet has been linked to mutations in reverse transcriptase.

Clinical Applications

Foscarnet is a well-established antiviral option in immunocompromised patients, and it is usually administered as a second-line option to ganciclovir. Foscarnet is an effective treatment for CMV retinitis, either as primary therapy or when there is suspicion or proof of ganciclovir resistance. Foscarnet is also useful for acyclovir-resistant mucocutaneous HSV or VZV infection, described mainly in patients with HIV infection and some hematology-oncology patients (175–179). One comparative trial of foscarnet and ganciclovir found that the two drugs were equivalent with respect to CMV outcome, but the foscarnet group exhibited an improved survival rate, with foscarnet extending life by an average of 4 months (134). When used as maintenance therapy for established CMV retinitis, the time to recurrence is inversely proportional to the foscarnet dosage employed. Therefore, a maintenance dosage of 120 mg/kg/day results in a delayed time to progression compared to that obtained with 90 mg/kg/day (180). Switching to foscarnet is recommended for CMV disease in transplant recipients if higher-level ganciclovir resistance emerges or if CMV disease is severe and low-level ganciclovir resistance is present or suspected. Uncontrolled studies suggest a benefit for foscarnet in visceral CMV disease, except for CMV pneumonitis in bone marrow transplant and lung transplant recipients (124). Foscarnet can be used safely in conjunction with ganciclovir given either concomitantly in full doses for severe syndromes, such as CMV ventriculoencephalitis in patients with AIDS or in alternating maintenance regimens

(181). Intravitreal foscarnet has been employed in CMV retinitis and acute retinal necrosis due to herpesvirus infection (76).

Foscarnet may have a particular role in the prophylaxis of CMV infections in hematopoietic stem cell transplant (HSCT) recipients. In allogeneic HSCT patients with CMV antigenemia or viremia, preemptive foscarnet therapy demonstrated an efficacy similar to that of ganciclovir but was associated with a lower rate of neutropenia and treatment-limiting hematologic toxicity (182). Although foscarnet exhibits *in vitro* and *in vivo* anti-HIV activities, its toxicity profile and requirement for parenteral administration preclude its use. It has been ineffective for HBV.

A topical cream containing 1% foscarnet was effective in the treatment of mucocutaneous HSV infections unresponsive to acyclovir (183) and avoids the adverse effects associate with intravenous administration. However, topical foscarnet creams are not commercially available.

Cidofovir

Cidofovir, (S)-1-[3-hydroxy-2-(phosphonylmethoxy)propyl] cytosine (Vistide) (Fig. 1; Tables 1 and 2), is an acyclic phosphonate nucleotide analog, which belongs to a family of phosphonylmethoxyalkyl derivatives of purines and pyrimidines.

Spectrum of Activity

Cidofovir has been primarily developed as an anti-CMV agent, but it has broad spectrum antiherpesvirus activity in addition to *in vitro* activity against human papillomaviruses, adenoviruses, poxviruses, and polyomaviruses. The *in vitro* IC_{50} for clinical CMV isolates is in the range of 0.1 μg/ml (184). In general, ganciclovir- and foscarnet-resistant strains of CMV and HSV remain susceptible to cidofovir.

Mechanism of Action

Cidofovir is taken up by both virally infected and uninfected cells and does not require the action of a virus-induced kinase to be converted to its active moiety (185). The structure of cidofovir represents a monophosphorylated nucleotide. This highly charged phosphonate poorly penetrates cell membranes, limiting its activity and contributing to renal toxicity. Cellular enzymes convert cidofovir to its diphosphate form, which, as an analog of a nucleotide triphosphate, is a competitive inhibitor of the viral DNA polymerase and causes premature chain termination. Cidofovir diphosphate has an intracellular $t_{1/2}$ of >48 h, an important factor that permits intermittent dosing schemes not previously possible for other anti-CMV agents. There is a 25- to 50-fold selectivity of cidofovir diphosphate for the viral, compared to the cellular, DNA polymerase (185).

Pharmacokinetics

Cidofovir can be administered intravenously, topically, and by ocular implant (no longer available). Direct intraocular injection of cidofovir is contraindicated. Plasma C_{max} averages 3.1 to 23.6 μg/ml with intravenous doses of 1.0 to 10.0 mg/kg, respectively. Cidofovir has poor CSF penetration, and ocular penetration has not been well characterized. The elimination $t_{1/2}$ in plasma is 2.6 hours, and 90% of the drug is excreted in the urine, with renal tubular secretion contributing substantially to clearance (186). Dose reduction is necessary, in the setting of renal insufficiency, if the benefit of the drug is thought to outweigh the risk of worsening renal function.

Adverse Effects

The major adverse effect of cidofovir is nephrotoxicity, which appears to result from renal tubular damage (187). At a dosage of 5 mg/kg twice weekly without concomitant probenecid, renal toxicity is common and is manifested by proteinuria and glycosuria (187). Concomitant use of high-dose probenecid appears to permit 5 mg/kg weekly or biweekly dosages of cidofovir to be tolerated. Cardiomyopathy occurred in one HIV-infected subject treated simultaneously with stavudine, and erythematous cutaneous eruptions have been described in subjects treated with cidofovir and probenecid (187). Iritis and hypotony (decreased intraocular pressure) are notable complications of both systemically and intravitreally administered cidofovir (188). Topical corticosteroids are effective in controlling the iritis associated with cidofovir if it is deemed in the patient's best interest to continue the drug. Neutropenia occurs in approximately 20% and requires monitoring. When cidofovir is administered with probenecid, hypersensitivity reactions, nausea, vomiting, and diarrhea occur commonly. Preclinical studies indicate that cidofovir has mutagenic, gonadotoxic, embryotoxic, and teratogenic effects. Cidofovir is associated with tumors in rats.

Drug Interactions

Probenecid has a nephroprotective effect in animal models and likely in humans. At cidofovir doses of 3 mg/kg, probenecid does not affect the clearance of cidofovir. However, at higher doses of cidofovir, probenecid given pre- and post-infusion reduces renal clearance (189). This differential effect of probenecid, dependent on the cidofovir dose, is not fully explained but has been hypothesized to reflect the presence of multiple tubular secretion pathways (189). The first may be high-affinity, probenecid-insensitive, and saturable. Once saturated, a probenecid-sensitive secretory mechanism may then be operative. Coadministration of cidofovir with probenecid is recommended to reduce nephrotoxicity. Coadministration with other nephrotoxic drugs, such as foscarnet or aminoglycosides, should be avoided.

Resistance

CMV resistance to cidofovir may emerge during chronic use. After 3 months of cidofovir therapy for CMV retinitis, 29% of subjects had CMV isolates with reduced susceptibility to cidofovir (190). However, reduced susceptibility to cidofovir *in vitro* may not predict clinical progression (191). Most of the known mutations that confer cross-resistance between ganciclovir and cidofovir map to the exonuclease domains located between amino acids 300 and 545 of UL54 (192). HSV and HHV-6 resistance to cidofovir emerges under drug-selection pressure *in vitro* but has not been reported in clinical isolates (30).

Clinical Applications

Cidofovir is effective in some HIV-infected patients with CMV retinitis and failure to respond or intolerance to ganciclovir and foscarnet. An induction course of cidofovir of 5 mg/kg weekly for 2 weeks is followed by dose-frequency reduction to every other week. In patients with relapsed CMV retinitis, the 5-mg/kg-every-other-week maintenance regimen was more effective in delaying recurrence but was associated with a higher incidence of nephrotoxicity than the lower-dose regimen (3 mg/kg) (193). Cidofovir may be considered in transplant recipients with CMV infections resistant to ganciclovir and foscarnet, although there is little

information on the efficacy of cidofovir in this setting, and dose-limiting nephrotoxicity is frequent (128).

Cidofovir can also be considered for the treatment of acyclovir-resistant and foscarnet-resistant mucocutaneous HSV infections, either by the intravenous route or topically, as a gel, which is not available as a commercial formulation (194–198). Clinical experience with the use of cidofovir for the treatment of drug-resistant VZV disease is very limited (199). Cidofovir, in addition to antiretroviral therapy, is not effective for the treatment of HIV-associated progressive multifocal leukoencephalopathy (200). Cidofovir is commonly used for the treatment of adenoviral infection in bone marrow and solid organ transplant recipients, although no randomized trials have been performed (201, 202). Cidofovir 1% ointment (not commercially available) has also been studied as an investigational drug for the topical treatment of human papillomavirus infections in immunosuppressed patients and for intralesional treatment of respiratory papillomatosis (203, 204).

Cidofovir interferes with poxviral DNA replication at concentrations well below those that are toxic for human cells and has a remarkably long intracellular $t_{1/2}$, making it an attractive agent for prophylaxis against smallpox were it to be utilized in a bioterrorism attack. Aerosolized doses of cidofovir prevented lethal intranasal or aerosol cowpox virus infection in murine models (205). Cidofovir may be clinically useful against other poxvirus diseases, including recalcitrant molluscum contagiosum and in immunosuppressed populations (206).

Other Antiherpesvirus Agents

Docosanol

Docosanol, $C_{22}H_{46}OC$ (Abreva), is a 22-carbon saturated alcohol that inhibits replication of HSV-1 and -2. A highly lipophilic compound, docosanol inhibits entry of lipid-enveloped viruses into target cells by fusion with the cell plasma membrane. As a result, the virus remains on the cell surface and is blocked from entering the nucleus for viral replication. Docosanol lacks direct virucidal activity (207), and cellular metabolism may be required for its antiviral activity. Topical docosanol was approved by the FDA in July 2000 for the treatment of recurrent herpes labialis in adults and children aged 12 years and older. It is the only nonprescription drug available for this indication. However, topical acyclovir is more effective than docosanol for treating HSV infections. Mild side effects, including headache and application site reactions, have been reported with docosanol.

Imiquimod

Imiquimod, 1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-4-amine (Aldara), is an agonist for Toll-like receptor 7 (TLR-7). Binding promotes maturation of dendritic cells as well as production of IFN-*a*, IFN-γ (indirectly), interleukin 12 (IL-12), IL-6, IL-8, and/or tumor necrosis factor from dendritic and other innate immune cells. There is no direct antiviral effect.

Imiquimod is available as a 5% topical cream. Following application to affected skin, some systemic absorption is seen, with levels in serum ranging from 0.1 to 3.5 ng/ml, depending on the dose and location of application. Urinary recoveries of as much as 0.15% are seen following thrice-weekly treatment with 75 mg for 16 weeks. Use of imiquimod is often limited by local toxicity, which, although usually mild or moderate, can be severe (skin weeping, erosion). Common adverse events include erythema, burning, pain, erosion, induration, and edema.

Imiquimod is approved for the treatment of actinic keratosis, superficial basal cell carcinoma, and external genital or perianal warts. Because of its immunomodulatory and consequent antiviral effects, it has been studied for treatment of both oral and genital HSV infection. Several case reports have noted more rapid healing of primary HSV-2 lesions and decreased viral shedding in patients with acyclovir-resistant HSV lesions. In an open-label, controlled study for treatment of herpes labialis, subjects receiving imiquimod had a longer time to recurrence, but the trial was stopped early due to severe local toxicity in the imiquimod group (208). A controlled dose-ranging study for herpes genitalis found no effect of imiquimod on time to recurrence (209).

Investigational Antiherpesvirus Agents

There are several novel and potentially effective compounds under preclinical and early clinical investigation, some of which are listed in Table 3. New antiherpesvirus drugs include inhibitors of immediate early-gene expression, nonnucleoside DNA polymerase inhibitors, helicase-primase inhibitors, inhibitors of protein-protein interactions among DNA replication proteins, and inhibitors of assembly, encapsidation, and nuclear egress (192, 210).

Brincidofovir

The lipophilic derivative of cidofovir, brincidofovir, hexadecyloxypropyl-cidofovir ([HDP-CDV] (CMX001) (Fig. 2), shows much improved oral bioavailability, increased cellular uptake, and lack of nephrotoxicity. Brincidofovir is absorbed in the small intestine and transported throughout the body as a phospholipid; it penetrates cell membranes more readily than cidofovir and is converted intracellularly to cidofovir diphosphate after cleavage of its lipid moiety and phosphorylation by cellular kinases (211). Brincidofovir achieves higher levels of intracellular cidofovir diphosphate than cidofovir and is highly active against many human

TABLE 3 Selected investigational antiherpesvirus drugs

Agent	Chemical backbone	Spectrum	Mechanism(s) of action	Stage of development
ODE-CDV[a]	Alkoxyalkyl ester of cidofovir	CMV, smallpox virus	Inhibition of DNA synthesis	Preclinical
Pritelivir (BAY 57-1293; AIC316)	Thiazole urea derivative	HSV-1, HSV-2	Helicase-primase inhibitor	Phase II
Amenamevir (ASP2151)	Oxadiazdiazolephenyl derivative	HSV-1, HSV-2, VZV	Helicase-primase inhibitor	Phase III
BILS 179 BS	Thioazolylphenyl compound	HSV-1, HSV-2	Helicase-primase inhibitor	Preclinical
Valomaciclovir (EPB 348, H2G)	Prodrug of acyclic guanosine analog	HSV-1, HSV-2, EBV, VZV	Nucleoside analog	Phase II

[a]ODE-CDV, octadecyloxyethyl-cidofovir.

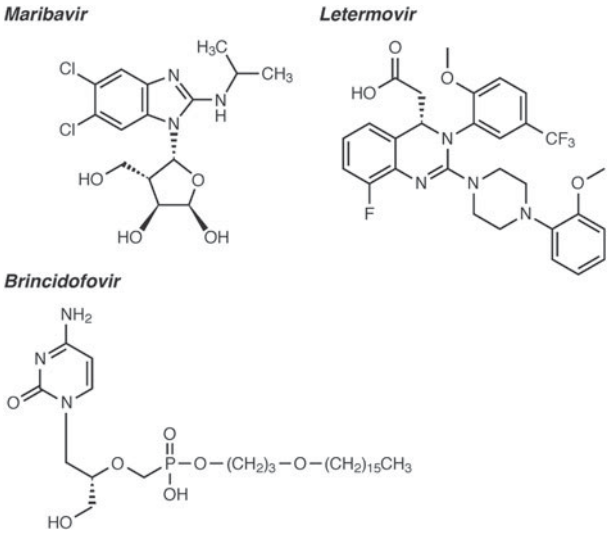

FIGURE 2 Chemical structures of investigational antiherpesvirus agents. (Reprinted with permission from reference [230]).

DNA viruses (212, 213). Brincidofovir was shown to suppress CMV disease in hematopoietic cell transplant in a phase 2 clinical trial (214). Myelosuppression or nephrotoxicity was not increased in the brincidofovir group. Diarrhea was a dose-limiting adverse effect; however, a dose of 100 mg twice weekly was effective at preventing CMV infection and resulted in milder diarrhea. A pivotal phase 3 clinical trial in hematopoietic stem-cell transplant recipients was recently terminated for the lack of efficacy. Preliminary results from a phase 3 study evaluating brincidofovir for the treatment of adenovirus infection showed a reduction or clearance of adenovirus during therapy (215). Brincidofovir has shown a survival benefit in an animal model of smallpox infection (216). *In vitro* studies have demonstrated activity against Ebola virus, an RNA virus, which is probably attributable to cellular toxicity. A subsequent clinical trial was halted by the manufacturer, citing an insufficient number of patients (217, 218). In contrast to DNA viruses, activity against Ebola virus requires the lipid moiety, and phosphorylation of cidofovir to the diphosphate form appears unnecessary (218). Brincidofovir is available for compassionate use.

Maribavir

Maribavir, 5,6-dichloro-2-(isopropylamino)-1-ß-L-ribofuranosyl-1H-benzimidazole (GW1263W94) (Fig. 2), belongs to a new class of drugs, the benzimidazole ribosides, and has a novel mechanism of action that differs from those of available CMV drugs. Maribavir does not require intracellular activation, and its phosphorylated forms do not directly inhibit the viral DNA polymerase. Instead, maribavir's main mechanism is related to blocking the nuclear egress of newly formed CMV virions by inhibiting UL97's phosphorylation of Lamin A/C (219, 220). Maribavir has activity against only two of the human herpesviruses, CMV and EBV. It is an oral agent with a plasma elimination $t_{1/2}$ of 3 to 5 hours and is extensively metabolized to an inactive metabolite by the hepatic cytochrome P450 3A4 isozyme (221).

A phase II dose-ranging study for CMV prophylaxis in allogeneic stem-cell transplant recipients compared maribavir 100 mg twice daily, 400 mg once daily, and 400 mg twice daily, with placebo. Within the first 100 days after transplantation, the incidence of CMV infection was lower in each maribavir dosing group (222). Two subsequent Phase 3 prophylaxis studies, one in allogeneic stem-cell transplant recipients (223) and one in liver transplant recipients (224), were conducted utilizing the 100 mg twice-daily dose. In stem-cell transplant recipients, maribavir failed to prevent CMV disease compared to placebo. The study in liver transplant recipients, which was stopped early, found that maribavir was inferior to oral ganciclovir. Failure in these studies has largely been attributed to the use of the lower 100 mg twice-daily dose (225). Maribavir has *in vitro* activity against ganciclovir- or cidofovir-resistant CMV, and small case series suggest a possible clinical benefit at higher doses in patients who failed to respond to other therapies and/or had known ganciclovir-resistant CMV (226). In all of these studies maribavir was well tolerated, with taste disturbance being the most significant adverse effect noted (223). An additional Phase II study for treatment of resistant or refractory CMV infections in transplant recipients, with doses as high as 1200 mg twice daily, has recently been completed (clinicaltrials.gov NCT00002373). Maribavir may be available for compassionate use if specific criteria are met.

Letermovir

The CMV terminase complex is comprised of two proteins, pUL89 and pUL56, whose main function is to cleave CMV DNA concatemers into functional CMV monomers (227, 228). Letermovir (AIC246, MK-8228) (Fig. 2) is a 3,4-dihydro-quinazoline-4-yl-acetic acid derivative and the most advanced viral-terminase complex inhibitor. Letermovir has activity only against CMV and, because of its novel mechanism, has no cross-resistance with other approved anti-CMV agents. A Phase II study in allogeneic hematopoietic-cell transplant recipients found oral letermovir effective in reducing the incidence of CMV infection compared to placebo and without any significant adverse effects (229). In this study, the highest dose of 240 mg/day had the greatest anti-CMV activity. A phase 3 study for prevention of CMV in allogeneic hematopoietic stem-cell transplant recipients examining intravenous or oral letermovir 240 mg or 480 mg once daily (dose dependent on concomitant administration of cyclosporin) is ongoing (clinicaltrials.gov NCT02137772). Letermovir is not available for compassionate use.

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Anti–Hepatitis Virus Agents

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13

Antiviral therapy for hepatitis viruses, in particular hepatitis C virus (HCV), has evolved dramatically over the past 5 to 10 years. This chapter reviews agents that have been, or are being, developed to treat hepatitis B virus (HBV) (Chapter 32) and HCV (Chapter 54) infections. Detailed information is provided for approved agents and those in advanced stages of clinical development. Agents in earlier stages of development are described more briefly. Agents approved but rarely if ever used for a specific pathogen have been removed from this edition (e.g., interferon-α and HBV). The reader is referred to the respective disease-specific chapters for full discussions of the viral agents and the diseases they cause.

ANTIVIRAL AGENTS FOR HEPATITIS B INFECTION

Lamivudine

Clinical Use

Lamivudine's (4-Amino-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-2(1H)-pyrimidinone; Epivir, Epivir-HB) mechanism of action against HBV parallels its mechanism against HIV. Lamivudine inhibits HBV DNA polymerase-reverse transcriptase (Table 1). For a full discussion of the pharmacology of lamivudine, refer to Chapter 11 on antiretroviral agents. Treatment with 100 mg of lamivudine daily for 12 months normalizes the serum alanine aminotransferase (ALT) level and improves histologic inflammatory scores in 50% to 70% of chronic hepatitis B patients (1). A loss of HBeAg is seen in up to 30% of patients treated with lamivudine, and HBeAg seroconversion, defined as the loss of HBeAg, undetectable levels of serum HBV DNA, and the appearance of antibodies against HBeAg, is seen in up to 20% of patients (2). HBeAg seroconversion is associated with durable antiviral responses. For several years, lamivudine was recommended for the initial treatment of individuals with chronic HBV infection who had active virus replication and active liver disease, defined as a positive serum HBV DNA and elevated serum ALT level with evidence of moderate to severe hepatitis on liver biopsy. With growing concern about lamuvidine resistance (see below) and the development of alternative agents with higher barriers to resistance, monotherapy with lamivudine is now discouraged. Lamivudine is effective in the treatment of IFN-*a*2b nonresponders and in the prophylaxis of HBV recurrence in liver transplant patients (3, 4). The combination of lamivudine with IFN-*a* for the treatment of chronic HBV infection has been disappointing, with most trials showing no long-term clinical benefit (5, 6). Lamivudine has also been studied in combination with other antiviral agents such as adefovir and telbivudine but has not shown significant clinical benefit when compared to currently recommended monotherapy options (7, 8). In HIV-negative patients, lamivudine has been used at 100 mg daily; however, it is recommended that HIV-infected patients receive the standard 150-mg-b.i.d. or 300-mg-twice daily dosage utilized for the treatment of HIV in combination with other antiretroviral agents. Such regimens should also include two agents active against HBV to try to prevent the emergence of HBV resistance.

Resistance

Resistance of HBV to lamivudine develops with prolonged therapy and is a major impediment to successful treatment. The frequency of genotypic resistance mutations increases from 24% of isolates after the first year to 67% after 4 years of therapy (9). Even higher rates of mutation have been seen in HIV- and HBV-coinfected patients given HIV treatment doses of lamivudine for long periods. The predominant lamivudine resistance mutations in HBV-infected patients are found in the catalytic domain of HBV polymerase in the tyrosine (Y), methionine (M), aspartate (D), aspartate (D) motif, principally M204I or M204V. These mutations appear to decrease viral replication competence, but a frequently associated mutation, L180M, may restore viral fitness (10). Although withdrawal of therapy results in repopulation with wild-type virus, retreatment with lamivudine results in rapid emergence of resistant strains (11). Failure to achieve a >1-$\log_{10}$ drop in HBV viral load after 3 months of therapy is associated with the emergence of lamivudine resistance.

Emtricitabine

Emtricitabine (4-Amino-5-fluoro-1-[(2S,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-2(1H)-pyrimidinone; Emtriva)

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TABLE 1 Key information on anti-HBV medications

Medication	Usual dosing	Mode of elimination	Considerations for renal impairment	Considerations for hepatic impairment	Major toxicities
Lamivudine	100 mg once daily HIV coinfection: 150 mg twice daily OR 300 mg once daily	Renal	Safe in mild, moderate, or severe renal impairment and in hemodialysis (HD) with appropriate dose reduction	None	Abdominal pain, nausea, vomiting
Emtricitabine	200 mg once daily	Renal	Safe in mild, moderate, or severe renal impairment and in HD with appropriate dose reduction	None	Dizziness, headache, diarrhea, rash, weakness, cough, rhinitis, anemia, neutropenia
Adefovir	10 mg once daily	Renal	Dose reduction recommended for CrCL 30–50 ml/min and hemodialysis. Not recommended for CrCl <30 ml/min	None	Rash, pruritus, abdominal pain, nausea, diarrhea, renal impairment
Entecavir	Nucleoside naïve: 0.5 mg once daily Lamivudine experienced: 1 mg once daily	Renal	Safe in mild, moderate, or severe renal impairment and in HD with appropriate dose reduction	None	Hepatitis, headache, dizziness, hematuria
Telbivudine	600 mg once daily	Renal	Safe in mild, moderate, or severe renal impairment and in HD with appropriate dose reduction	None	Abdominal pain, elevated creatinine kinase, headache, nasopharyngitis, upper respiratory infection, malaise/fatigue, lactic acidosis, hepatitis
Tenofovir disoproxil (TDF)	300 mg once daily	Renal	Dose reduction recommended for CrCL < 50 mL/min. If TDF is potentially the cause of renal impairment alternative therapy should be considered	None	Diarrhea, nausea, renal impairment, weakness

(Figure 1; Table 1) is a 5′-fluorinated analogue of lamivudine with the same mechanism of action and potency (see Chapter 11 on antiretroviral agents). Although not United States Food and Drug Administration (FDA) approved for the treatment of HBV, several studies have demonstrated the clinical efficacy of emtricitabine in chronic HBV infection. As with lamivudine, resistance develops rapidly when the drug is used as monotherapy.

Clinical Use

Nucleoside/tide analogue naïve chronically infected HBV patients treated with 48 weeks of emtricitabine 200 mg daily have superior histologic improvement and loss of detectable HBV DNA compared to placebo. Resistance to emtricitabine was observed in 13% of patients (12). No studies have compared emtricitabine to lamivudine but neither agent appears to offer a clinically relevant benefit compared to the other. Emtricitabine has a minimal role as monotherapy in the treatment of chronic HBV but may be used when coformulated with tenofovir.

Adefovir Dipivoxil

Adefovir dipivoxil (bis-pivaloyloxymethyl-9-(2-phosphonylmethoxyethyl) adenine; Hepsera) is the orally bioavailable prodrug of adefovir, an adenine nucleotide analog.

Spectrum of Activity

Adefovir dipivoxil has activity against several DNA and RNA viruses, including retroviruses, herpesviruses, and hepadnaviruses. It was initially developed for use against HIV, but this use was abandoned because of safety concerns at the dosing levels needed to inhibit HIV. It retains activity against HBV, however, at doses that are not associated with excessive toxicity. The EC_{50} of adefovir for HBV is approximately 0.003 μg/ml, making it 10-fold more potent than tenofovir but half as potent as lamivudine *in vitro* (13). Because of its low oral bioavailability, however, adefovir is inferior to both lamivudine and tenofovir at doses used clinically.

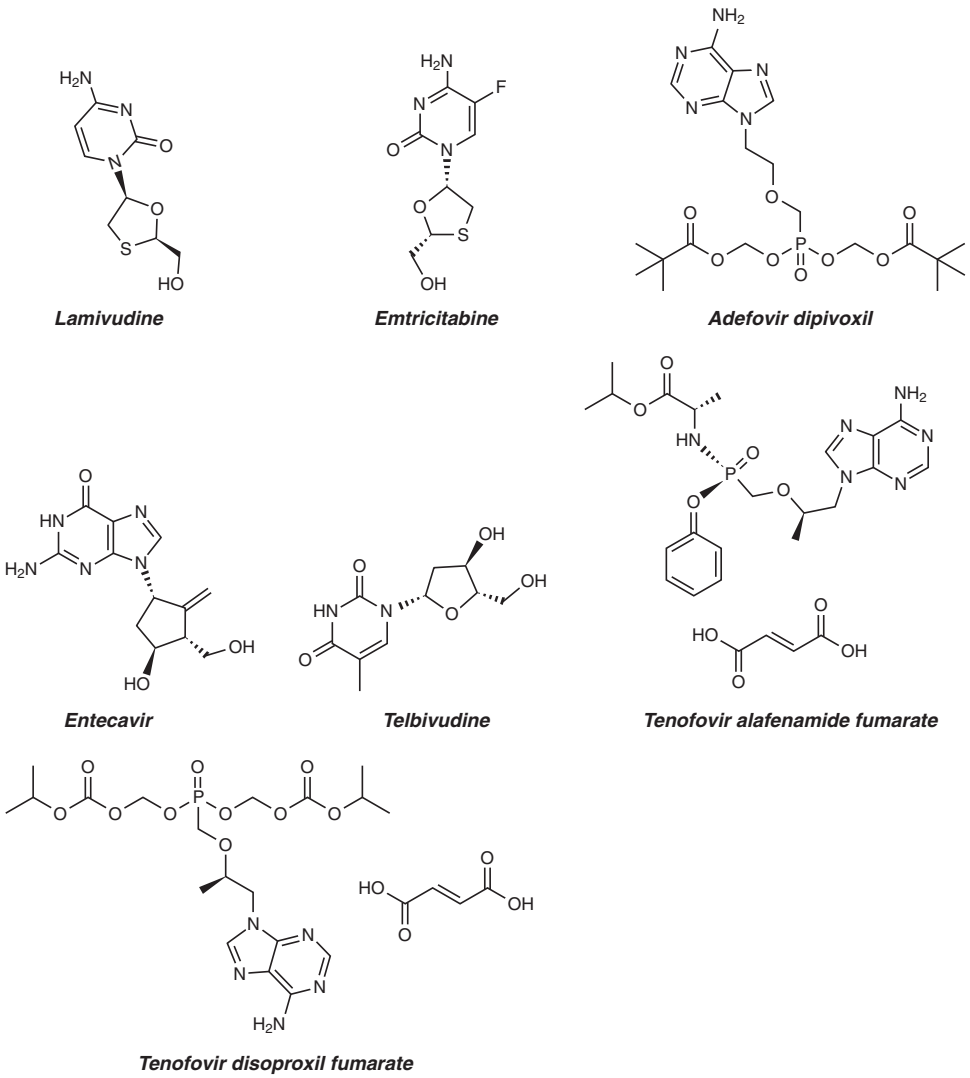

FIGURE 1 Chemical structures of agents with activity against hepatitis B virus.

Mechanism of Action

Once absorbed, adefovir dipivoxil is converted to adefovir, which then undergoes two intracellular phosphorylation steps. The active intracellular metabolite of adefovir, 9-[2-(phosphonomethoxy)ethyl]adenine (PMEA) diphosphate, inhibits viral DNA polymerases by competing with dATP and terminating the DNA chain (14).

Pharmacology

Absorption of adefovir does not appear to be affected by food or stomach pH. About 10% of the absorbed dose is converted intracellularly to the active form, the long elimination half life ($t_{1/2}$) (18 h) of which allows for once-daily dosing. Adefovir is not metabolized by cytochrome P450 enzymes. The activity of adefovir appears to be unaffected or enhanced when the drug is combined with other anti-HBV agents.

Adverse Effects

The toxicities of adefovir at the doses used for treating HBV have been primarily gastrointestinal and hepatic, including nausea and transient increases in transaminase levels. At the higher doses used to treat HIV, gastrointestinal disturbances, elevated liver enzyme levels, and delayed renal effects, including renal tubular dysfunction mimicking the Fanconi syndrome, occurred (15).

Resistance

Resistance to adefovir develops more slowly than resistance to lamivudine, with approximately 29% of patients demonstrating resistance after 5 years of adefovir monotherapy. Of note, only 20% of the patients had evidence of viral rebound and 11% had elevations in ALT (i.e., clinical resistance) (16). Several mutations are associated with adefovir resistance, including N236T and A181V (16). Although these mutations decrease susceptibility by only 3% to 15% *in vitro*, hepatitis flares and decompensation are seen (17). Primary adefovir resistance has been associated with the I233V mutation, but viruses with this mutation do retain susceptibility to tenofovir and entecavir (18). Adefovir is a potential option for treating lamivudine-resistant HBV.

Clinical Applications

Adefovir is effective for the treatment of chronic HBV infection in both HBeAg-positive and HBeAg-negative

subjects. After 48 weeks of treatment with adefovir at 10 mg daily, the mean decrease in HBV DNA in HBeAg-positive subjects was 3.52 $\log_{10}$ copies/ml (19). Results were similar in the trial of HBeAg-negative subjects (20). Adefovir therapy also resulted in greater improvements in liver histology, decreases in ALT, and, in subjects who were HbeAg positive at the start of the study, higher rates of HBeAg seroconversion. Because of the substantial rates of resistance seen with prolonged adefovir therapy, however, the use of adefovir monotherapy is generally discouraged. The addition of lamivudine to adefovir therapy appears to reduce the emergence of adefovir resistance both in those with lamivudine resistance and in those who have never received treatment (8, 21). Tenofovir is preferred to adefovir due to its more potent antiviral activity and superior safety.

Entecavir

Entecavir (2-amino-1,9-dihydro-9-[(1*S*, 3*R*, 4*S*)-4-hydroxy-3-(hydroxymethyl)-2-methylenecyclopentyl]-6*H*-purin-6-one, monohydrate; Baraclude) is a guanine nucleoside analog (Figure 1; Table 1).

Spectrum of Activity

Entecavir possesses potent and selective activity against hepadnaviruses. In cell culture, it is between 30- and 1,000-fold more active than lamivudine (22). Preclinical studies originally suggested that entecavir possessed no anti-HIV-1 activity, but *in vivo* activity resulting in an approximately 1-$\log_{10}$ reduction in plasma HIV-1 RNA level in humans has been described (see "Resistance" below) (23).

Mechanism of Action

After it is phosphorylated, entecavir interferes with multiple functions of HBV polymerase, including priming, reverse transcription, and DNA-dependent DNA synthesis (24).

Pharmacology

With once-daily dosing, steady state is achieved after 6 to 10 days. In healthy volunteers receiving 0.5 mg daily for 14 days, the plasma C_{max} averaged 4.2 ng/ml and the elimination $t_{1/2}$ was 130 h (25). The intracellular $t_{1/2}$ for entecavir triphosphate is approximately 15 h. Oral administration of entecavir with a high-fat meal or a light meal results in significant delays in absorption, decreases in C_{max}, and decreases in area under the curve (AUC). Consequently, entecavir should be administered on an empty stomach. Entecavir is primarily eliminated unchanged by the kidneys, and the dosage should be adjusted in subjects with a creatinine clearance of less than 50 ml/min. Coadministration of entecavir with adefovir, lamivudine, or tenofovir results in no significant pharmacokinetic interactions (26). No interactions with other medications have been reported.

Adverse Effects

The adverse effects most frequently associated with entecavir in clinical trials were headache and upper respiratory infection, and these were generally mild. Elevations in liver enzymes occur less frequently than with lamivudine monotherapy (27, 28).

Resistance

Entecavir resistance rarely emerges in subjects with no prior nucleoside exposure, even after 2 years of monotherapy. Interestingly, although the M204V/I mutation associated with lamivudine resistance does not confer resistance to entecavir, it appears to be a prerequisite for such resistance to emerge. When combined with one of the lamivudine resistance mutations (M204V/I and L180M), three known mutations confer entecavir resistance (T184A/I/S, S202C/G, and M250I/L), although alone they are insufficient to cause such resistance (29). In a study of lamivudine-refractory subjects treated with entecavir for 2 years, viral rebound occurred in 1% of subjects after 1 year and 9% after 2 years (29).

In preclinical testing, entecavir had no reported anti-HIV activity and was therefore used to treat HIV- and HBV-coinfected patients who did not yet need treatment for their HIV. However, HIV coinfected individuals have developed an M184V mutation in HIV reverse transcriptase while on entecavir monotherapy (23). Entecavir should not be prescribed to HIV coinfected individuals who are not on a completely suppressive antiretroviral regimen.

Clinical Applications

Entecavir monotherapy has been compared to lamivudine in two large trials of HBeAg-positive and HBeAg-negative subjects with elevated ALT, detectable HBV DNA, and evidence of inflammation on liver biopsy. After 52 weeks, entecavir was superior to lamivudine for the frequency of histologic improvement (72% versus 62% in HBeAg-positive subjects (27) and 70% versus 61% in HBeAg-negative subjects (27). Entecavir was also superior to lamivudine in frequency of undetectable HBV DNA (67% versus 36% in HBeAg-positive subjects and 90% versus 72% in HBeAg-negative subjects). Compared to adefovir, entecavir was associated with a greater reduction of HBV DNA after treatment for 52 weeks. The mean change in HBV DNA was significantly greater with entecavir (-6.23 versus -4.42 $\log_{10}$ copies/ml) (30). Despite the resistance issues noted above, entecavir has been shown to be effective in HBeAg-positive subjects who are refractory to lamivudine (31), although a dose increase to 1 mg daily is recommended for patients with known resistance to lamivudine. However, entecavir is generally not recommended in those with lamivudine resistance unless they are unable to tolerate tenofovir. It may be used in those with resistance to adefovir unless they have previously been treated with lamivudine (32). Long-term entecavir therapy for 5 years has been associated with normalized aminotransferase levels and a decrease in inflammation and fibrosis scores (33).

Telbivudine

Telbivudine (1-(2-Deoxy-β-L-erythro-pentofuranosyl)-5-methyl-2,4(1H,3H)-pyrimidinedione; Tyzeka) is a nucleoside analog approved for the treatment of chronic HBV infection.

Spectrum of Activity

Telbivudine is a competitive inhibitor of the HBV DNA polymerase. Telbivudine has no activity against any human viruses other than HBV and does not inhibit human cellular polymerases. Inhibition of human mitochondrial DNA polymerase γ is felt to be the cause of much of the toxicity seen with other nucleoside analogs (e.g., neuropathy, myopathy, and lactic acidosis) (34).

Mechanism of Action

Telbivudine requires biotransformation to the triphosphate form, but unlike lamivudine, it preferentially inhibits anti-complementary (second-strand) DNA (35). Because it lacks a 3′OH group, it acts as a chain terminator once incorporated into the growing DNA strand.

Pharmacology

In pharmacokinetic studies, C_{max} following an oral dose of telbivudine occurs in 0.8 to 2.8 h (36) and ranges from 0.20 to 5.46 μg/ml, with an AUC of approximately 26 μg/ml•h. Telbivudine is cleared primarily via the kidneys and elimination is biphasic, with an intracellular $t_{1/2}$ of approximately 14 h and an elimination $t_{1/2}$ of approximately 40 h. Dose adjustments should be made in patients with a creatinine clearance of <50 ml/min.

Adverse Effects

Telbivudine is generally well tolerated. Elevations in serum creatinine kinase have been more common in those receiving telbivudine than in those receiving lamivudine, although these were not associated with muscle-related adverse events (37). The package insert for all nucleoside analogs carries a boxed warning for lactic acidosis, though this has not yet been reported with telbivudine specifically.

Resistance

Resistance to telbivudine has developed at less than half the rate of resistance to lamivudine (5% versus 11% for HBeAg-positive subjects and 2.3% versus 10.7% for HBeAg-negative subjects) (37). The signature mutation associated with telbivudine resistance has been an M204I substitution in the HBV DNA polymerase (38). Telbivudine is active against strains with an isolated M204V mutation but inactive against strains with M204V plus L180M. *In vitro*, telbivudine remains active against strains resistant to adefovir.

Clinical Applications

One large study comparing telbivudine to lamivudine monotherapy showed that telbivudine is not inferior to lamivudine after 52 weeks (38). In the subgroup of HbeAg-positive subjects, telbivudine was superior to lamivudine for the primary endpoint (viral load of <5 $\log_{10}$ copies/ml coupled with normalization of ALT or loss of HBeAg), and both HBeAg-positive and HBeAg-negative subjects achieved greater and faster reductions in HBV DNA if they were receiving telbivudine. A 2-year follow-up study found that telbivudine was superior to lamivudine for the same primary endpoint in both HBeAg-positive and -negative subjects (39). Rates of resistance were also lower in the telbivudine group (25% versus 40%). When compared directly with adefovir in HBeAg-positive subjects, telbivudine treatment for 52 weeks resulted in a greater mean reduction in HBV DNA than adefovir treatment for 52 weeks or 24 weeks of adefovir followed by 28 weeks of telbivudine (40). Subjects in the telbivudine-only arm were also significantly less likely to experience primary treatment failure (2% versus 29% in the adefovir-only arm).

Tenofovir Disoproxil Fumarate

Tenofovir disoproxil fumarate (Bis{[(isopropoxycarbonyl) oxy]methyl} ({[(2R)-1-(6-amino-9H-purin-9-yl)-2-propanyl] oxy}methyl)phosphonate (2E)-2-butenedioate; TDF, Viread) is an oral prodrug of tenofovir, a nucleotide analog. TDF is approved for the treatment of both HIV and chronic HBV infections, and is the preferred agent for treating HBV in the context of HIV/HBV coinfection (41). For further details on TDF pharmacology, drug interactions, and adverse effects, see Chapter 11. It is a nucleoside inhibitor of the HBV polymerase and also acts to block protein priming. In HBV mono-infection, changing to TDF often results in excellent viral suppression in subjects resistant to lamivudine who have viral breakthrough on adefovir (42). A large study examining nucleoside-naïve HBeAg-positive subjects found that TDF 300 mg daily for 48 weeks resulted in more frequent reduction of HBV DNA levels of <400 copies/ml when compared to adefovir (43). The efficacy of TDF was comparable to adefovir in HBeAg-negative subjects. TDF is also the preferred agent for those with prior exposure to lamivudine, with a retrospective study showing 100% treatment response at an average follow-up of 23 months (44).

Studies comparing TDF alone to combination therapy of TDF with emtricitabine have generally found little benefit to the addition of emtricitabine. In one randomized study of patients with HBV mutations associated with lamivudine resistance, rates of virologic suppression at 96 weeks were comparable between the monotherapy and combination therapy groups, suggesting that emtricitabine does not enhance the antiviral activity of TDF in this context (45). Another study comparing TDF monotherapy and TDF in combination with emtricitabine in patients who did not achieve adequate virologic suppression on adefovir resulted in similar rates of viral suppression (84% versus 82%) in the combination therapy and monotherapy arms (46). Further, viral kinetic curves were reported as identical between the two groups. In a separate trial of treatment-naïve patients with high initial viral loads and normal ALT levels, HBV DNA levels were undetectable in 55% of patients who received TDF monotherapy and 76% of those who received TDF in combination with emtricitabine (47). However, viral kinetic curves were similar between the two groups, HBeAg seroconversion and hepatitis surface antigen loss were rare in either group, and no participant in either group developed tenofovir-resistant virus. Although it has been speculated that monotherapy may predispose to the development of minor variant quasispecies, this was not seen in a small study that compared the development of quasispecies in subjects receiving TDF monotherapy to those receiving combination therapy with emtricitabine (48). Further, resistance of HBV to TDF has not yet been shown to develop, even with long-term use (49). Therefore, TDF monotherapy remains the preferred strategy for management of chronic HBV in the absence of HIV at the present time. This may well change as more data emerge.

Tenofovir Alafenamide Fumarate

Tenofovir alafenamide fumarate (Isopropyl N-[(S)-({[(2R)-1-(6-amino-9H-purin-9-yl)-2-propanyl]oxy}methyl)(phenoxy) phosphoryl]-L-alaninate (2E)-2-butenedioate (2:1); TAF) (Figure 1), a novel prodrug of tenofovir, is currently FDA approved for the treatment of HIV infection in multiple fixed-dose combinations (and is in phase 3 trials for the treatment of chronic HBV infection). Please refer to Chapter 11 for a detailed review of pharmacology, drug interactions, and adverse events. It results in much lower plasma levels of tenofovir and provides higher intracellular levels of active drug allowing lower daily doses and decreased renal toxicity compared to TDF. In a study of patients coinfected with HIV and chronic HBV who had been virologically suppressed on antiretrovirals with activity against HBV, subjects were switched to a single tablet formulation of elvitegravir 150 mg, cobicistat 150 mg, emtricitabine 200 mg, and TAF 10 mg. Through week 48 of treatment, HIV and HBV viral suppression was maintained in the same proportion of participants, confirming acceptable activity of TAF against HBV (50).

ANTIVIRAL AGENTS FOR HEPATITIS C VIRUS INFECTION

Unlike HIV and HBV, which have persistent DNA reservoirs in infected cells even with effective antiviral suppression, complete cure of HCV infection is possible with medical therapy. In clinical studies of anti-HCV medications, an undetectable HCV viral load 12 or 24 weeks after the end of treatment (termed sustained virologic response [SVR] 12 or 24) has been considered a proxy for cure. Before 2011, the standard of care for treatment of HCV was pegylated interferon plus ribavirin, a regimen that led to SVR24 only slightly more than 50% of the time and was often associated with moderate or worse toxicity (51, 52). Development of anti-HCV direct-acting agents (DAAs), medications with direct anti-HCV activity, began in earnest in the early 2000s following the development of the HCV replicon, in which key components of the HCV genome could be made to replicate *in vitro*, allowing for the study of inhibitory compounds (53). At around the same time, detailed crystal structures of the key viral proteins became available, allowing for rational modeling and development of antiviral compounds. The first approved DAAs, boceprevir, and telaprevir, received regulatory approval in 2011 in combination with pegylated interferon and ribavirin. The number of anti-HCV agents in clinical development and with regulatory approval has increased dramatically in recent years. As of 2016, interferon-free regimens are the standard of care for treatment of all genotypes of HCV. Figure 2 presents a timeline outlining key events in the development of modern HCV therapies.

Table 2 gives an overview of the key characteristics of DAA-based regimens approved for treatment of HCV infection by the U FDA or with regulatory approval expected within the 2016 calendar year. Medications in earlier stages of clinical development are listed in Table 3. As new agents are being approved at a rapid pace, the reader is referred to hcvguidelines.org for the most up to date information on drug and regimen approvals.

Interferons

Interferons (IFNs) are part of the repertoire of human cytokines that have important multifunctional capabilities, including antiviral, immunomodulatory, and antiproliferative effects. There are currently nine approved IFN preparations for clinical use, of which five are approved for the treatment of chronic HCV. The nomenclature for this group of cytokines has evolved over the past four decades, although the term interferon stems from the original discovery of these compounds, which interfered with viral replication. There are two major types of human IFNs that have been studied clinically: IFN-*a*/*β* (leukocyte/fibroblast) and IFN-*γ*. There are over 12 subtypes of IFN-*a* but only 1 IFN-*β* subtype and 1 IFN-*γ* subtype. It is not clear why an array of closely related IFN-*a* subtypes exist, but one postulate is that the redundancy ensures that this natural defense system remains operative.

Spectrum of Activity

IFNs possess inhibitory activity against a broad array of DNA and RNA viruses. IFN-*a* and -*β* typically have greater antiviral activity than does IFN-*γ*, and the subspecies of IFN-*a* may express differential activities against a particular viral agent.

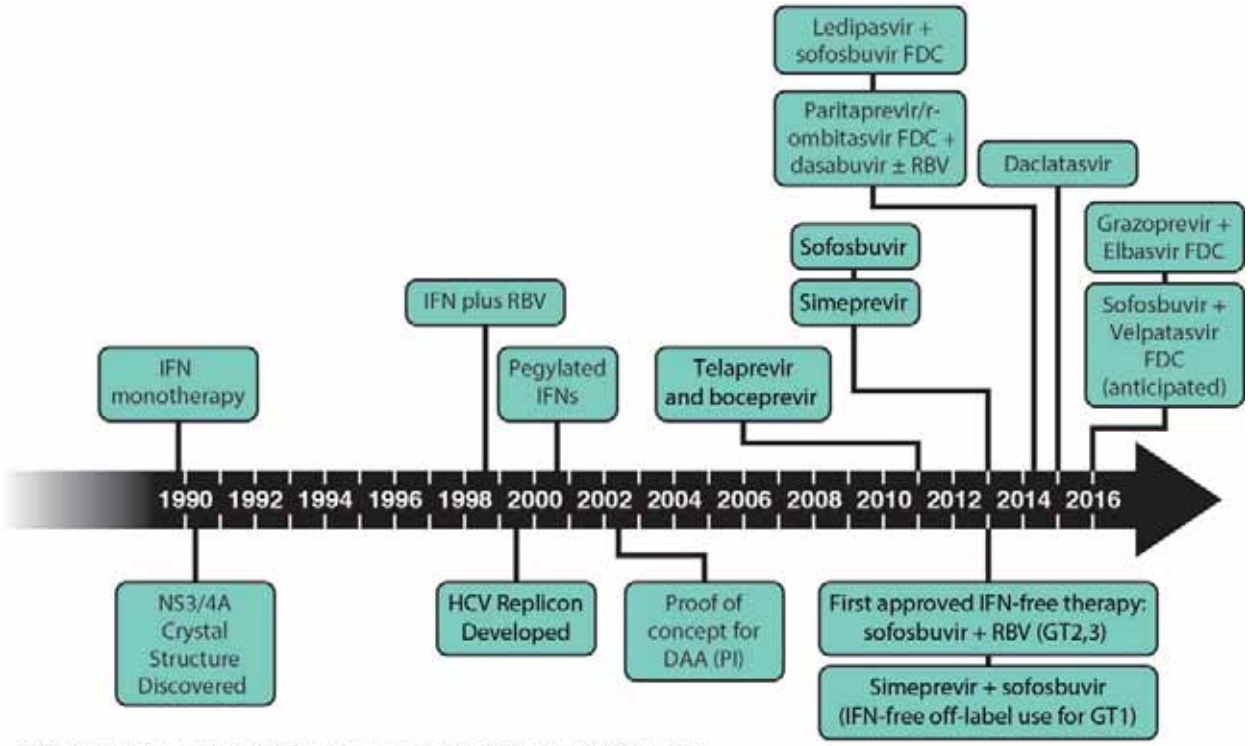

FIGURE 2 Timeline of key historical events in the development of anti-HCV direct-acting agents.

TABLE 2 Key information on anti-HCV DAA regimens

Regimen	Class	Standard Dose	FDA Approved Genotype	Duration	Mode of Elimination	Considerations in Renal Impairment	Considerations in Hepatic Impairment	Major Toxicities
Ombitasvir/ paritaprevir/ ritonavir FDC + dasabuvir	Ombitasvir: NS5A inhibitor Paritaprevir: NS3/ 4A inhibitor Dasabuvir: NS5B inhibitor (non-nucleoside) ritonavir: CYP3A4 inhibitor	ombitasvir 12.5 mg/ paritaprevir 75 mg/ ritonavir 50 mg FDC two tablets once daily plus dasabuvir 250 mg twice daily	1	Genotype 1a without cirrhosis: 12 weeks + RBV Genotype 1a with cirrhosis: 24 weeks + RBV Genotype 1b without cirrhosis: 12 weeks Genotype 1b with cirrhosis: 12 weeks + RBV	Hepatic	Safe in mild, moderate, or severe renal impairment No data in HD Care must be taken if ribavirin coadministered	CTP A: Safe CTP B: Not recommended CTP C: Contraindicated	Fatigue, nausea, rash, pruritus, insomnia, LFT elevations
Ombitasvir/ paritaprevir/ ritonavir FDC	see above	ombitasvir 12.5 mg/ paritaprevir 75 mg/ ritonavir 50 mg FDC two tablets once daily	4	12 weeks + RBV	Hepatic	see above	see above	see above
Sofosbuvir	NS5B inhibitor (nucleoside)	400 mg daily	1–4	Genotype 1: In combination with ledipasvir, simeprevir, or daclatasvir as below Genotype 2: In combination with RBV x12 weeks. May consider expanding to 16 weeks or more in patients with cirrhosis. May consider in combination with daclatasvir in patients with contraindication to RBV but data is limited. Genotype 3: In combination with daclatasvir as below, OR for 12 weeks with RBV and peg-IFN OR for 24 weeks with RBV alone (IFN ineligible) Genotype 4: See FDC with ledipasvir below	Renal	Mild/moderate: no dose adjustment Severe (CrCl < 30 ml/min or HD): No safe dose established	None	Fatigue, headache

(*Continued*)

TABLE 2 Key information on anti-HCV DAA regimens (*Continued*)

Regimen	Class	Standard Dose	FDA Approved Genotype	Duration	Mode of Elimination	Considerations in Renal Impairment	Considerations in Hepatic Impairment	Major Toxicities
Sofosbuvir/ ledipasvir FDC	Sofosbuvir: NS5B inhibitor (nucleoside) Ledipasvir: NS5A inhibitor	sofosbuvir 400 mg/ ledipasvir 90 mg FDC once daily	1	Treatment-naïve: 12 weeks (may give 8 weeks if baseline VL <6 million) Treatment-experienced, noncirrhotic: 12 weeks Treatment-experienced with cirrhosis: 24 weeks, +/-RBV	Renal	See sofosbuvir	None	Fatigue, headache, insomnia, and nausea
Simeprevir	NS3/4A inhibitor	150 mg daily	1	Used in combination with sofosbuvir No cirrhosis: 12 weeks Cirrhosis: 24 weeks (+/- RBV)	Hepatic	None	CTP A: Safe CTP B: Not recommended CTP C: Not recommended	Hyperbilirubinemia Photosensitivity Rash
Daclatasvir	NS5A inhibitor	30–90 mg daily (depending on drug interactions)	3	Used in combination with sofosbuvir No cirrhosis: 12 weeks Cirrhosis: 24 weeks (+/- RBV)	Hepatic	None	None	Fatigue, headache
Grazoprevir/ Elbasvir FDC	Grazoprevir = NS3/4A inhibitor Elbasvir= NS5A inhibitor	grazoprevir 100 mg/ elbasvir 50 mg FDC once daily	1 and 4	12–16 weeks +/- RBV based on genotype, baseline NS5A polymorphisms, treatment history	Hepatic	Safe in ESRD, including HD	CTP A/B: Safe CTP C: No data	Headache, fatigue, nausea, LFT elevations
Velpatasvir/ sofosbuvir FDC	Ledipasvir: NS5A inhibitor	sofosbuvir 400 mg/ velpatasvir 100 mg FDC once daily	Pending, see text	12–24 weeks +/- RBV based on genotype, cirrhosis status, and treatment history	Hepatic	None	None	Headache, fatigue, nasopharyngitis, and nausea

CrCl, creatinine clearance; CTP, Child-Turcotte-Pugh; ESRD, end-stage renal disease; FDC, fixed-dose combination; HD, hemodialysis; IFN, interferon; LFT, liver function tests; RBV, ribavirin.

TABLE 3 Other anti-HCV drugs in phase 1 and 2 not in text

Mechanism	Drug	Stage of Development	Comments
NS5A Inhibitors			
	Odalasvir	Phase 2	Being studied with ACH-3422 for genotype 1
			Has activity in viruses with resistance to first-gen. NS5A inhibitors
	Samatasvir	Phase 2	Pan-genotypic
			Low SVR4 rates when combined with simeprevir and ribavirin
	MK-8408	Phase 1	Pan-genotypic
			Being studied as potential FDC with grazoprevir and MK-3682 in genotype 1 and 3 HCV
	TD-6450	Phase 1	Heterodimeric structure designed to increase potency against virus with key NS5A RAVs
NS5B Inhibitors			
	Mercitabine	Phase 2	
	MK-3682	Phase 2	Uridine-based nucleoside prodrug
			Being studied as potential FDC with grazoprevir and MK-8408 in genotype 1 and 3 HCV
	TMC647055	Phase 2	Nonnucleoside, macrocyclic compound that binds to "thumb pocket 1" binding site
	VX-135	Phase 2	Uridine-based nucleoside prodrug
	ACH-3422	Phase 1	Uridine-based nucleoside prodrug
			High barrier to resistance, pan-genotypic
			Being studied with odalasvir for genotype 1
	AL-335	Phase 1	Nucleoside NS5B inhibitor
			Being studied in combination with odalasvir and simeprevir for genotype 1
	GSK2878175	Phase 1	Nonnucleoside inhibitor
			To be studied in combination with RG-101
NS3/4A Inhibitors			
	Danoprevir	Phase 2	Highly effective with pegIFN/RBV for genotype 1b. Unclear role for future all DAA combinations
	Sovaprevir	Phase 2	High SVR12 rates seen in genotype 1b patients when combined with odalasvir and ribavirin; GT1a patients developed NS3 RAVs
Other			
	Miravirsen	Phase 2	Antisense oligonucleotide that targets miR122, a hepatic microRNA necessary for HCV replication.
	RG-101	Phase 2	Anti-miR122 antisense oligonucleotide conjugated to GalNac to allow for possible single-visit therapy (injectable)
			Being studied in combination with numerous DAAs
	BIT225	Phase 2	Novel mechanism of action; Inhibitor of p7 protein, small membrane protein required for HCV replication
	SB 9200	Phase 1	Immune modulatory medication that activates RIG-1 and NOD2, regulators of interferon signaling in response to HCV
			May move forward in combination with DAAs

Mechanisms of Action

IFNs are synthesized in response to a wide variety of infections and stimuli of the innate immune system and are an integral component of the complex cytokine network. Nearly all cell types can produce IFN-*a* and -*β*, while IFN-γ is produced largely by T and natural killer (NK) cells. Once produced, IFNs possess no inherent antiviral activity, but act by inducing an antiviral state within target cells. IFNs bind to specific receptors on the cell surface: IFN-*a* and -*β* have the same receptor. These receptors are composed of at least two subunits that must be present to ensure that full IFN action is induced (54). Following receptor binding, receptor-associated tyrosine kinases (Tyk2 and JAK1 for IFN-*a* and -*β* and JAK1 and JAK2 for IFN-γ) are activated and, in turn, phosphorylate specific cytoplasmic proteins (termed STAT proteins). These proteins move to the nucleus, where they bind to specific *cis*-acting elements in the promoter regions of IFN-inducible genes (55). Transcription of these genes occurs within minutes of IFNs' binding to the cell receptors.

Depending upon the viral agent and cell type, IFNs can inhibit viral replication at nearly all steps from penetration to particle release. Several mechanisms of IFN-induced antiviral activity have been described: (i) 2′-5′ oligoadenylate synthetases are activated by double-stranded (viral) RNA and are responsible for the conversion of ATP into a series of oligonucleotides designated 2′,5′-oligo(A)s. The 2′,5′-oligo

(A)s then activate an RNase, RNase L, which can cleave single-stranded mRNAs (56). (ii) A double-stranded RNA-dependent protein kinase termed PKR (P68 kinase, P1, DAI, dsl, and eukaryotic initiation factor 2 [eIF2] kinase) is activated by double-stranded (viral) RNA and autophosphorylated, which in turn, phosphorylates the alpha subunit of eIF2. This prevents the recycling of eIF2 with inhibition of protein synthesis (57). (iii) The induction of a phosphodiesterase inhibits peptide chain elongation. (iv) MxA protein, which binds to cytoskeletal proteins and inhibits viral transcriptases, is synthesized (58). (v) Nitric oxide synthetase is induced by IFN-γ in macrophages (59).

Although some of the IFN-inducible functions have been linked to the inhibition of replication of individual viruses, for the most part there is uncertainty in trying to dissect out single activities of IFNs in this process, since more than one mechanism may be operative simultaneously. In addition, some antiviral effects of IFNs result indirectly from stimulation of antiviral immune functions. For example, the immunomodulatory activities of IFNs, such as induction of cytotoxic T-cell and NK cell activity and the induction of major histocompatibility complex proteins, may also help control viral infections (see Chapter 16).

Pharmacology

For treatment of chronic HCV, IFNs must be administered intravenously, intramuscularly, or subcutaneously. The elimination $t_{1/2}$ of IFN-*a* in plasma following intravenous administration is 2 to 3 h, which is extended to 4 to 6 h following intramuscular or subcutaneous administration, with 80% of an administered dose absorbed following injection by the latter routes. The levels achieved in plasma are dose proportional. The relevance of classic pharmacokinetic parameters of IFNs in relation to their antiviral activity is questionable, since systemic effects are measurable in the absence of detectable IFN levels in plasma. Two long-acting, slow-release formulations of IFN combined with polyethylene glycol (pegylated) are currently available (PegIntron and Pegasys). These products can be administered subcutaneously once per week and achieve sustained levels in blood equivalent to those of standard IFN given three times a week. Penetration into CSF and respiratory secretions is minimal.

Adverse Effects

IFNs cause a broad range of side effects, most commonly systemic and hematologic. Dose-related influenza-like symptoms are common with initiation of treatment and generally include fever, chills, headache, nausea, myalgias, and arthralgias. Gradual dose escalation may be helpful; tolerance to these side effects can develop with time, but this is not uniform. The most common hematologic side effects are leukopenia and thrombocytopenia, and thus treatment of immunocompromised hosts or patients taking other myelotoxic agents can be difficult. Other important side effects include neurotoxicity (including somnolence, confusion, electroencephalographic changes, behavioral changes, and seizures), psychiatric disturbances (especially depression), hepatotoxicity, nephrotoxicity, thyroiditis, and alopecia.

Drug Interactions

Few formal drug interaction studies with the approved preparations of IFNs have been performed. Care must be exercised when coadministering other potentially myelosuppressive or neurotoxic drugs. Pegylated IFN-*a*2a and -*a*2b (collectively referred to hereafter as "peg-IFN" unless specified otherwise) inhibit cytochrome P450 (CYP) 1A2 enzymes, but do not affect the pharmacokinetics of drugs metabolized by CYP2C9, CYP2C19, CYP2D6, or CYP3A4 hepatic microsomal enzymes. No studies of drug interactions have been published with IFN-β.

Resistance

Many viruses have developed strategies to evade or inhibit IFN-mediated antiviral effects. However, acquired resistance to the actions of IFNs through specific mutations during therapeutic administration has not been well documented. Variability in the response of chronic HCV infection to IFN therapy has been associated with amino acid substitutions in the NS5A protein, although the significance of this association remains unclear. Specifically, heterogeneity between codons 2,209 and 2,248 may affect the ability of IFN to bind to double-stranded-RNA-dependent protein kinase, thereby reducing the host antiviral response (60). In HCV replicon cell culture models, IFN-resistant HCV strains have decreased expression and activity of Tyk2 and JAK1, two proteins in the JAK-STAT pathway that are intermediates in IFN-mediated intracellular signaling (61).

Clinical Applications

IFN-*a* preparations are beneficial in the treatment of chronic HBV and HCV infections, although responses are not uniform and relapse rates after discontinuation of treatment are substantial. The response to IFN therapy in patients with chronic hepatitis C is higher than in patients with hepatitis B and depends on numerous host factors, most importantly IL28B genotype (62, 63), but also age, stage of liver disease, ethnicity, and hepatocyte HLA antigen expression. Virologic factors, including viral load and HCV genotype, also affect IFN responsiveness, with genotypes 2 and 3 having better rates of response to IFN than genotypes 1 and 4 (51, 64). Peg-IFN in combination with ribavirin achieves rates of viral response superior to those of standard IFN-ribavirin therapy and peg-IFN alone (51, 52) including in patients coinfected with HIV and HCV (64). Response rates were even higher when peg-IFN and ribavirin were given in conjunction with a third DAA, such as a protease inhibitor (telaprevir or boceprevir) or the NS5B inhibitor sofosbuvir (see below). However, given the newer DAA combinations available with shorter treatment duration, improved side effect profiles, and vastly superior response rates, IFN-based regimens are no longer the standard of care in almost all cases of HCV infection.

Ribavirin

Ribavirin (1-β-d-ribofuranosyl-1,2,4-triazole-3-carboxamide; Rebetol, Copegys, Ribapak, Ribasphere, Virazole) is a synthetic nucleoside analog of guanosine approved in the United States for the treatment of RSV infection and in combination with IFN-*a* for the treatment of HCV infection.

Spectrum of Activity

Ribavirin has *in vitro* activity against a broad range of both RNA and DNA viruses, including flaviviruses, paramyxoviruses, bunyaviruses, arenaviruses, retroviruses, herpesviruses, adenoviruses, and poxviruses. In structure-activity studies, both the ribose moiety and the base are essential for maintenance of antiviral activity.

Mechanism of Action

The mechanism of action of ribavirin is incompletely understood and possibly multifactorial. Ribavirin is phosphorylated by host cell enzymes to ribavirin triphosphate. In the

case of influenza virus, ribavirin triphosphate interferes with capping and elongation of mRNA and may directly inhibit viral RNA polymerase activity. Some studies have suggested a direct antiviral effect of ribavirin on HCV, although the data are not consistent and this effect, if present, is probably small. Ribavirin inhibits HCV RNA polymerase, but only at concentrations exceeding those achieved clinically. Inhibition of inosine-5′-monophosphate dehydrogenase by ribavirin monophosphate depletes the intracellular pools of guanosine triphosphate required for RNA synthesis. Ribavirin may also act indirectly by immunomodulatory effects, causing a change in the host T-cell response from a T-helper 2 (Th2) to a Th1 response. For hepatitis C, the shift to a Th1 profile with production of Th1 cytokines, especially IFN-γ, is believed to halt virion production, increase lysis of infected hepatocytes, stop transformation to neoplastic cells, and limit fibrogenesis (65). Finally, ribavirin may act as a mutagen, increasing the error rate in replicating RNA strands and pushing the virus over the threshold of replicative incompetence. There is evidence to support this theory from HCV replicon studies, but human data remain inconclusive.

Pharmacology

Aerosol, oral, and intravenous formulations of ribavirin exist, but the aerosol and oral preparations are the only ones approved in the United States and only oral preparations are used to treat HCV infection. Concentration of ribavirin in plasma after a single oral dose has three phases: rapid absorption, rapid distribution, and a long elimination phase. Following a 600-mg dose, the C_{max} was 782 ng/ml, the elimination $t_{1/2}$ was 79 h, and the AUC was 13,394 ng|•|h/ml. Administration with food enhances absorption and results in a 70% increase in the concentration in plasma. The plasma to CSF ratio is approximately 0.7.

Adverse Effects

Oral and intravenous administration is associated with anemia, which is related to intravascular hemolysis and bone marrow–suppressive effects. Depression, pruritus, rash, nausea, and cough have been reported. When the drug is given as an aerosol preparation, bronchospastic reactions can occur, and ocular irritation has been reported. Ribavirin also has immunosuppressive effects. *In vitro*, antigen- and mitogen-driven lymphocyte proliferative responses are suppressed by ribavirin, and release of mast cell mediators may be inhibited. Ribavirin has teratogenic, carcinogenic, and mutagenic properties in preclinical assays, and therefore its use in pregnant women is contraindicated.

Drug Interactions

In vitro studies suggest that ribavirin can antagonize the activity of zidovudine by inhibiting its phosphorylation, although such an effect has not been observed *in vivo* (66). Conversely, ribavirin can potentiate the anti-HIV activity of didanosine by facilitating its phosphorylation, but there have been reports of severe mitochondrial toxicity resulting in fatal and nonfatal lactic acidosis in patients receiving didanosine and ribavirin concurrently (67). In patients coinfected with HIV and HCV, these drugs should not be used together but with improvements in the agents available to treat both infections, this question has become moot.

Resistance

Replicon studies with HCV Huh7 strains have demonstrated the appearance of the B415F-to-Y mutation in the NS5B RNA polymerase when exposed to ribavirin. Strains with the 415Y mutation have a replicative advantage over the 415F strains in the presence of ribavirin, but when ribavirin is removed, all strains revert to the 415F variant and demonstrate improved replicative capacity over the 415Y mutant. In another study, the G404S and E442G mutations in the NS5A protein were both associated with low-level ribavirin resistance (68). There have been no reports of RSV, influenza virus, or HIV isolates resistant to ribavirin.

Clinical Applications

Ribavirin is approved for the treatment of HCV in combination with a number of IFN-containing as well as IFN-free DAA regimens. Despite marked advances in response rates with DAA combinations, ribavirin is still recommended to be given with these agents in several of the most difficult to treat patient populations, such as treatment-experienced patients with cirrhosis.

Direct-Acting Agents: NS5B Inhibitors

NS5B is the HCV RNA-dependent RNA polymerase necessary for viral replication. There are two classes of NS5B inhibitors available or in development. The first class includes the nucleoside/nucleotide inhibitors, which bind at the active site of the polymerase and prevent chain elongation of the viral RNA. Because there is little variation in the enzymatic active site across the different HCV genotypes, and because any mutations in the active site tend to render the virus unfit to replicate efficiently, nucleoside/nucleotide NS5B inhibitors tend to have activity across a range of genotypes and tend to have a high barrier to resistance (69, 70). The second class is the nonnucleoside NS5B inhibitors, which bind outside of the active site and cause allosteric changes that hinder polymerase activity. Because there is less conservation of nonactive site amino acids across genotypes, these inhibitors are generally not pan-genotypic and have a lower barrier to resistance. Figure 3a shows the chemical structures of commonly prescribed NS5B inhibitors.

Sofosbuvir

Spectrum of Activity

Sofosbuvir ((S)-Isopropyl 2-((S)-(((2R,3R,4R,5R)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-fluoro-3-hydroxy-4-methyltetrahydrofuran-2-yl)methoxy)-(phenoxy)phosphorylamino)propanoate; Sovaldi) is active against HCV genotypes 1 through 6 with varying EC_{50} values. The EC_{50} values of sofosbuvir for genotypes 1a, 1b, 2a, 2b, 3a, 4a, 5a, and 6a range from 0.014 to 0.11 μM (71).

Mechanism of Action

Sofosbuvir, also known as GS-7977, is a monophosphate prodrug converted to its pharmacologically active triphosphate form (GS-461203) within hepatocytes. Its design was based upon experiments with older DAAs that suggested it would be possible to develop a monophosphate prodrug that could be delivered to hepatocytes where it would then be metabolized into highly active triphosphate. This design strategy amplifies the intracellular concentrations of the active metabolite to allow for lower oral doses, more favorable pharmacokinetics, increased potency, and the ability to coformulate the drug in fixed-dose combinations with other DAAs. After phosphorylation, the nucleotide analog is incorporated into HCV RNA via the NS5B polymerase resulting in premature chain termination and disruption of transcription of the viral polyprotein (70–73).

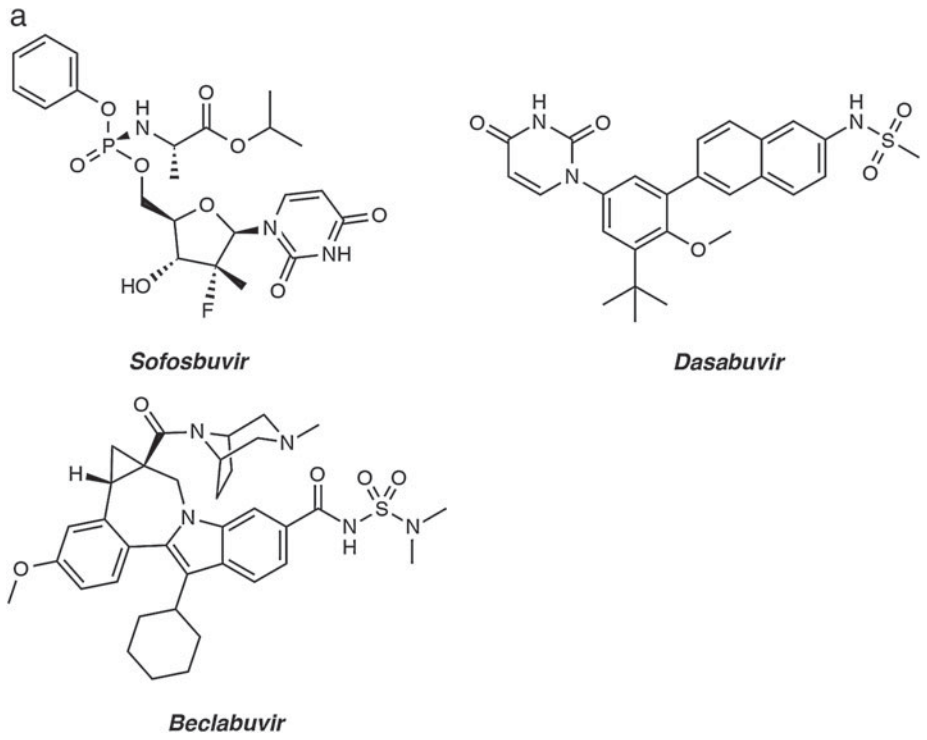

FIGURE 3a Chemical structures of HCV NS5B inhibitors.

Pharmacology

Sofosbuvir is absorbed rapidly, reaching peak plasma concentration in 0.5 to 2 hours. AUC_{0-24} is 969 ng•hr/ml for the parent compound sofosbuvir and 6,790 ng•hr/ml for the predominant inactive metabolite GS-331007, which accounts for >90% of drug-related systemic exposure. There is no difference in drug absorption when administered with a high fat meal or fasting conditions, so sofosbuvir can be taken without regard to meals. Sofosbuvir is metabolized in the liver through sequential hydrolysis, phosphoramidate cleavage, and subsequent phosphorlyation to its pharmacologically active nucleoside analog triphosphate form (71).

The major pathway of elimination is renal; 78% of drug is recovered in the urine as GS-331007. The half-lives of sofosbuvir and GS-331007 are 0.4 hours and 27 hours, respectively. Overall exposure of sofosbuvir and GS-331007 are increased in patients with mild renal impairment compared to patients with normal renal function. Dose adjustments are not needed in mild or moderate renal impairment, but sofosbuvir is not recommended to be given when CrCl is <30 ml/min as safety and efficacy have not been established in this setting. Overall exposure is also increased in moderate and severe hepatic impairment; however, this is not thought to be clinically significant (71).

Adverse Effects

The most common side effects associated with sofosbuvir are fatigue and headache, which occur in >20% of patients, and tend to occur more frequently in patients who receive longer courses of sofosbuvir and/or coadministered ribavirin. Other side effects include nausea, insomnia, pruritus, and anemia. However, these may be attributable to coadministered ribavirin. Discontinuation of sofosbuvir due to adverse events attributable to the drug has been rare in clinical trials (74–76).

Drug Interactions

Sofosbuvir is a substrate of the P-gp efflux pump and is not recommended to be used with P-gp inducers due to the risk of decreased sofosbuvir exposure. P-gp inducers include medications such as rifamycins and anticonvulsants like carbamazepine, phenytoin, phenobarbital, and oxcarbazepine. However, sofosbuvir can be used concomitantly with P-gp inhibitors, and it does not have interactions with drugs metabolized by the CYP 3A4 enzyme system (71).

Postmarketing analysis revealed cases of symptomatic bradycardia, including fatal cardiac arrest, when sofosbuvir, in combination with another DAA, was used concomitantly with the antiarrhythmic amiodarone. Onset of symptoms is usually hours to days after medication administration but may be delayed up to 2 weeks in some cases. Therefore, the combination of sofosbuvir and amiodarone is not recommended unless there are no available treatment alternatives (71, 77).

Resistance

Amino acid substitutions leading to reduced susceptibility to sofosbuvir have been identified in cell culture and clinical trials but their clinical significance is unknown. The sofosbuvir associated resistance substitution NS5B S282T confers 2- to 18-fold reduced susceptibility to sofosbuvir *in vitro*. However, this variant was absent at baseline and in all failure isolates in phase 3 clinical trials. Treatment emergent substitutions L159F, V321A, C316N, S282R, and L320F detected in phase 3 trials were inconsistently associated with treatment failure. Resistance-associated variants

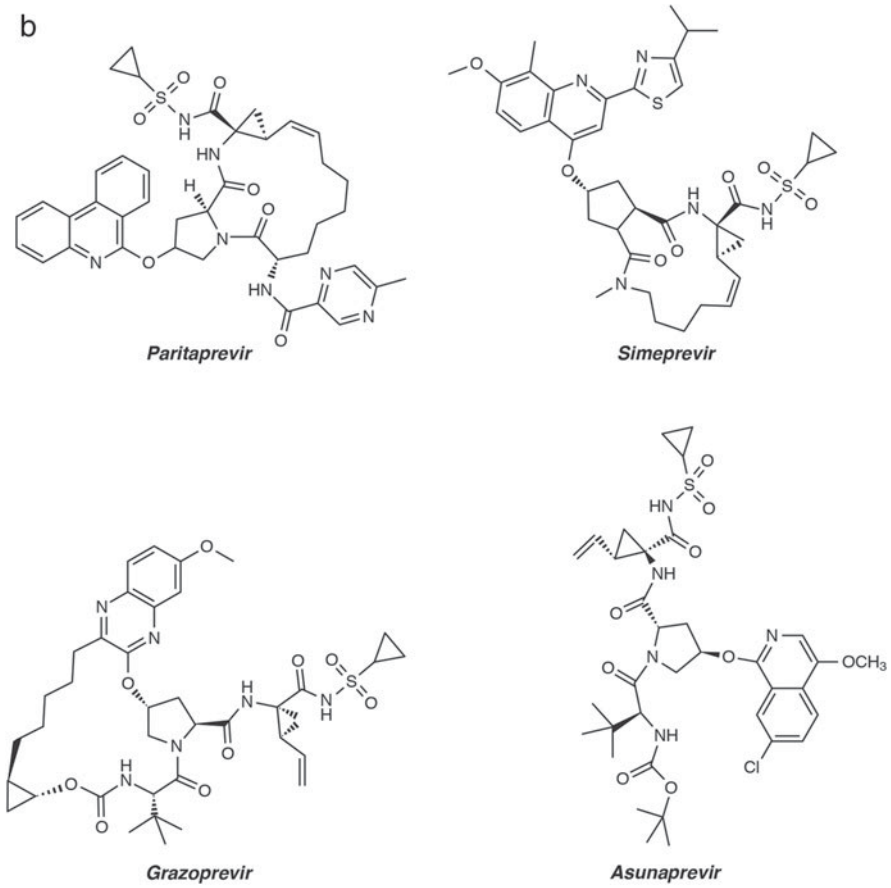

FIGURE 3b Chemical structures of HCV NS3/4A inhibitors.

(RAVs) associated with other DAAs appear to have a limited effect on susceptibility to sofosbuvir (71).

Clinical Applications

Sofosbuvir has been extensively studied in multiple combination regimens against HCV genotypes 1 through 6 and has revolutionized anti-HCV treatment. Early clinical studies of sofosbuvir in combination with peg-IFN and ribavirin in patients with genotype 1 led to SVR12 rates of 90% to 93% (78, 79). However, regimens containing peg-IFN and ribavirin are no longer recommended when alternatives are available. Sofosbuvir has been studied in IFN-free combinations with daclatasvir, ledipasvir, simeprevir, and velpatasvir. For detailed descriptions of the clinical trial results related to these regimens, please refer to the sections below pertaining to these agents.

Sofosbuvir with ribavirin, even in the absence of a second DAA or IFN, has been studied in patients with genotypes 2 and 3. A landmark trial showed identical SVR12 rates of 67% with sofosbuvir and ribavirin compared to peg-IFN and ribavirin (79). In patients for whom IFN is not an option, sofosbuvir and ribavirin for 12 weeks achieved overall SVR12 rates of 78% in patients with genotype 2 or 3 HCV infections; however, genotype 3 and cirrhosis were both negative predictors of response, with SVR12 rates of 93% versus 61% for patients with genotype 2 compared to genotype 3 and SVR12 rates of 81% versus 48% in patients without cirrhosis compared to patients with cirrhosis. Longer durations of therapy are needed for patients with genotype 3, especially in those with cirrhosis (80). With the advent of multiple ribavirin-free all-DAA regimens with activity against genotypes 2 and 3, it is likely that the regimen of sofosbuvir plus ribavirin will fall out of favor.

Dasabuvir

Spectrum of Activity

Dasabuvir (*N*-{6-[5-(2,4-Dioxo-3,4-dihydro-1(2*H*)-pyrimidinyl)-2-methoxy-3-(2-methyl-2-propanyl)phenyl]-2-naphthyl}methanesulfonamide) inhibited the activity of genotype 1 polymerases *in vitro* at IC_{50} ranging from 2.2 to 10.7 nM. It does not have inhibitory activity against polymerases from HCV genotypes 2 to 4 (81).

Mechanism of Action

Dasabuvir is a nonnucleoside inhibitor of the viral NS5B polymerase. Although the exact mechanism is unknown, based upon resistance data, it is believed to act mechanistically like the benzothidiazine NS5B inhibitors, binding to the palm I site of the enzyme and inhibiting RNA chain initiation allosterically (82–84).

Pharmacokinetics

Dasabuvir is available in 250 mg tablets and dosed twice daily. Absorption is increased by 30% when taken with food. Dasabuvir achieves peak levels approximately 4 hours after administration. The average plasma elimination $t_{1/2}$ in healthy volunteers is 9.2 hours (85). It is greater than 99.5% protein-bound. Dasabuvir is primarily excreted through the

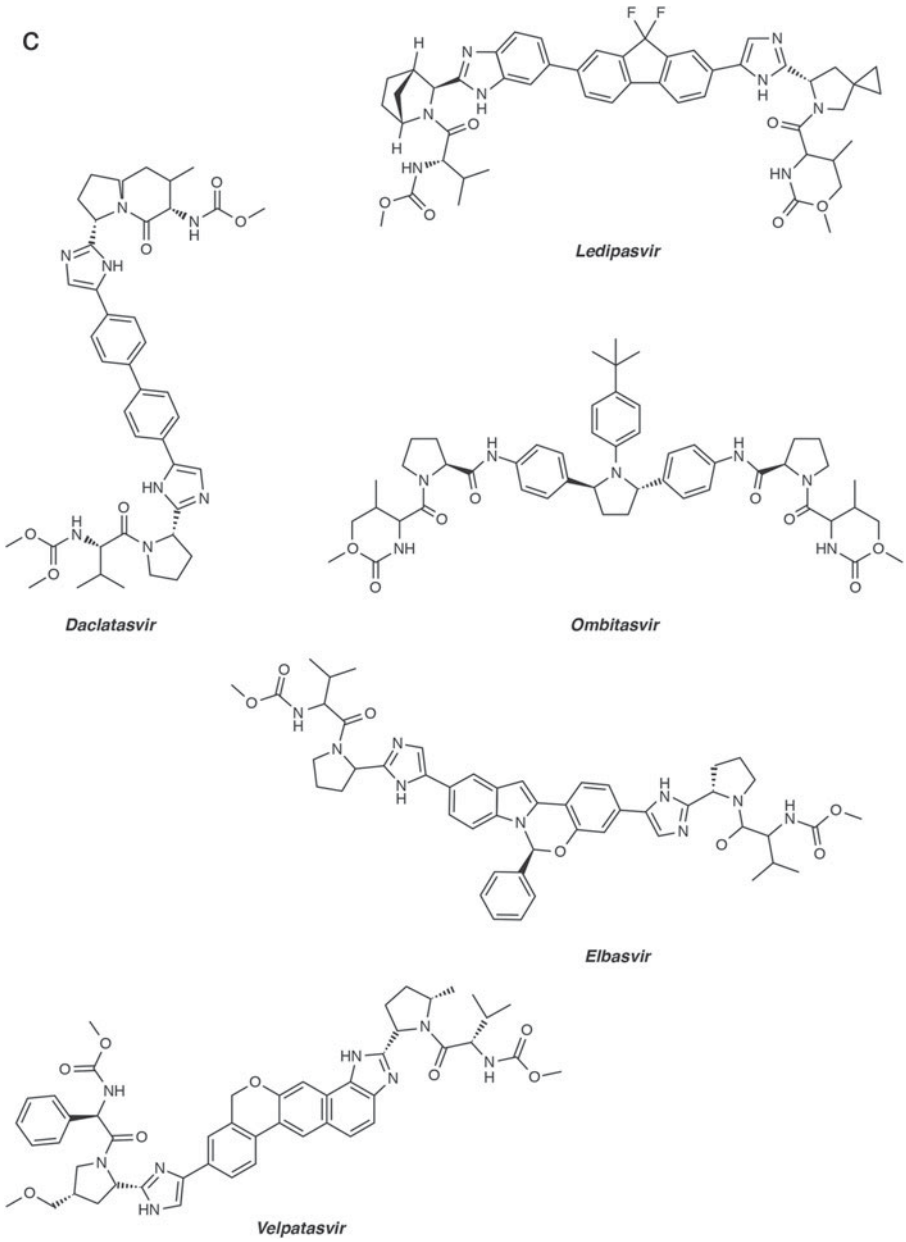

FIGURE 3c Chemical structures of HCV NS5A inhibitors.

biliary system into the feces, both as unchanged dasabuvir and as dasabuvir M1 metabolite (86). Urinary excretion is minimal. Dasabuvir and dasabuvir M1 AUC in subjects with Child-Pugh A or B cirrhosis is similar to that of subjects with normal hepatic function. However, in subjects with Child-Pugh class C cirrhosis, dasabuvir AUC is increased by over 300%, and dasabuvir M1 AUC is increased by 70% (85). It is therefore contraindicated in individuals with Child-Pugh class C cirrhosis.

Adverse Effects

There are minimal data on the adverse effects associated specifically with dasabuvir because in clinical studies, dasabuvir has always been coadministered with paritaprevir, ritonavir, and ombitasvir. For a detailed review of the adverse effects associated with this regimen, please refer to the ombitasvir section. In a clinical trial of ombitasvir, paritaprevir, and ritonavir, with or without ribavirin for genotype 4 HCV, rates of fatigue were lower than rates seen in most studies of the same regimen plus dasabuvir for genotype 1 HCV (87). Rates of other adverse events were comparable. Therefore, it is possible that the fatigue might have been attributed to the dasabuvir.

Drug Interactions

Dasabuvir is primarily metabolized by CYP2C8, which forms the basis for most of the clinically significant drug-drug interactions. There also may be some metabolism by CYP3A. Dasabuvir is an *in vitro* inhibitor and substrate of P-gp and breast cancer resistance protein (BCRP), although the clinical significance of this is uncertain (88). Coadministration with CYP2C8 inhibitors, such as gemfibrozil,

is associated with a significant increase in dasabuvir levels, and is contraindicated. For additional discussion of drug-drug interactions associated with coadministered ombitasvir, paritaprevir, ritonavir, and ribavirin, please refer to the sections pertaining to these medications.

Resistance

In a replicon model of HCV genotype 1a, the major RAVs selected by dasabuvir were S556G and C316Y, which conferred 30-fold and 1,472-fold resistance, respectively. In a similar model using genotype 1b virus, the primary variants selected were C316Y and M414T, which were associated with 1,569-fold and 470-fold resistance, respectively. *In vitro*, dasabuvir demonstrated wild-type levels of activity against variants with the S282T polymorphism, the main variant associated with resistance to nucleoside/nucleotide analogs (81). In clinical studies of patients with HCV genotype 1a, the S556G/S variant was the most common baseline variant associated with treatment failure and also the most common treatment-emergent RAV (89). The emergence of resistance was much rarer in individuals with genotype 1b.

Clinical Applications

Dasabuvir has only been studied in combination with coformulated ombitasvir/paritaprevir/ritonavir, with or without ribavirin for genotype 1 HCV. For a description of relevant clinical trials, please refer to the section on ombitasvir.

Beclabuvir

Spectrum of Activity

In phase 1 studies beclabuvir ([(1aR,12bS)-8-Cyclohexyl-N-(dimethylsulfamoyl)-11-methoxy-1a-{[(1R,5S)-3-methyl-3,8-diazabicyclo[3.2.1]oct-8-yl]carbonyl}-1,1a,2,12b-tetrahydrocyclopropa[d]indolo[2,1-a][2]benzazepine-5-carboxamide]), a nonnucleoside NS5B inhibitor, resulted in an approximately 2.5 $\log_{10}$ decline of HCV RNA in genotype 1a and 1b infected subjects within 24 hours after a single dose of 300 mg (90). In cell culture 50% EC_{50} values for beclabuvir were 3 nM and 6 nM for genotypes 1a and 1b, respectively (91). Similar values were observed for genotypes 3a, 4a, and 5a; however, activity versus genotypes 2 and 6a were variable.

Pharmacokinetics

The pharmacokinetics of beclabuvir were evaluated in combination with daclatasvir and asunaprevir (92). Using a dose of 75 mg twice daily, peak plasma concentrations were 1,375 ng/ml for beclabuvir and 318 ng/ml for its active metabolite BMS-794712; levels were comparable in cirrhotic patients. Peak concentrations were achieved in 2.5 to 3.0 hours. The AUC_{12} values observed at steady-state were 6,755 ng•h/ml and 1,174 ng•h/ml. Beclabuvir is metabolized to its equipotent metabolite, BMS-794712 via CYP3A4 (93). *In vitro* it has been shown to be a weak inducer of CYP3A4. In a phase 1 study, coadministration with beclabuvir resulted in mildly decreased exposure to midazolam (94). Beclabuvir in combination with asunaprevir and daclatasvir resulted in 32% and 38% decreases in C_{max} levels of escitalopram and sertraline, respectively (95).

Adverse Effects

Beclabuvir is being developed exclusively in fixed-dose combination with asunaprevir and daclatasvir. In clinical trials, this coformulation has been generally well tolerated. The most commonly reported adverse events have included headache, fatigue, asthenia, diarrhea, nausea, pruritus, and abdominal pain. In clinical trials, all adverse effects considered treatment-related were mild to moderate in intensity and did not lead to drug discontinuation (96, 97).

Resistance

In preclinical studies, the main NS5B RAVs associated with beclabuvir exposure were primarily at a single site (P495A/S/L/T) (91). The most common treatment emergent NS5B RAVs in patients with virologic failure in clinical trials of beclabuvir, in combination with daclatasvir and asunaprevir, were at NS5B amino acid 495, most notably P495L (96, 97).

Clinical Applications

In a phase 3 trial of noncirrhotic patients with genotypes 1a and 1b treated with the fixed-dose combination of beclabuvir, daclatasvir, and asunaprevir, SVR12 rates were 91.3% and 89.3% for treatment-naïve and treatment-experienced patients, respectively (97). In a separate trial, SVR12 rates were also high in patients with cirrhosis, regardless of prior treatment experience, and regardless of whether ribavirin was administered concomitantly (98). A small study also showed an SVR12 rate of 91.5% for this regimen in persons with genotype 4 HCV infection (99).

GS-9669

GS 5-(3,3-dimethylbut-1-ynyl)-3-[[4-hydroxy-4-[[(3S)-tetrahydrofuran-3-yl]oxymethyl]cyclohexyl]-[(1R)-4-methylcyclohex-3-ene-1-carbonyl]amino]thiophene-2-carboxylic acid) is a nonnucleoside inhibitor of the NS5B polymerase that binds at the allosteric thumb site II location. GS-9669 is active against HCV genotype 1 and is currently being studied as part of various combination regimens with other DAAs including sofosbuvir and ledipasvir (100). In a phase 2A trial, treatment-naïve patients treated with 6 weeks of ledipasvir, sofosbuvir, and GS-9669 achieved 95% SVR12 (101).

Direct-Acting Agents: NS3/4A Inhibitors

The NS3/4A serine protease is responsible for posttranslational processing of HCV and also functions in the evasion of the host innate immune response (102). NS3/4A interferes with signaling of RIG-1, a cellular helicase that binds HCV RNA, and activates factors that result in IFN-β production. NS3 also cleaves Cardiff, an additional factor downstream of RIG-1, to further decrease IFN-β production (103), and blocks Toll-IL-1 receptor domain-containing adaptor (TRIF), which normally serves as another activator for interferon regulatory factor 3 and NF-κB (104).

The first-generation NS3/4A inhibitors, boceprevir and telaprevir, were the first DAAs to gain regulatory approval in the United States, in 2011. Highlighting the rapid pace of HCV DAA development, manufacturing and distribution of both boceprevir and telaprevir have been discontinued in the United States as these agents have quickly been eclipsed by drugs with greater efficacy and fewer side effects. HCV protease inhibitors that either have regulatory approval or are expected to have approval soon are described in detail below, as are several agents in late phase 2 clinical development. Medications in earlier clinical development are summarized in Table 3. Figure 3b shows the chemical structures of NS3/4A inhibitors commonly used in clinical practice.

Simeprevir

Spectrum of Activity

Simeprevir (2R, 3aR, 10Z, 11aS, 12aR,14aR)-N-(cyclopropylsulfonyl)-2-[[2-(4-isopropyl-1,3-thiazol-2-yl)-7-methoxy-8-methyl-4-quinolinyl]oxy]-5-methyl-4,14-dioxo-2,3,3a,4,5,6,7,8,9,11a,12,13,14,14a-tetradecahydrocyclopenta

[c]cyclopropa[g][1,6]diazacyclotetradecine -12a(1H)-carboxamide; Olysio) has antiviral activity against HCV genotypes 1a, 1b, and 4. EC_{50} values for genotype 1a range from 23 to 28 nM and for genotype 1b range from 3.7 to 25 nM (105).

Pharmacology

Simeprevir is absorbed to its maximum concentration between 4 and 6 hours after oral administration. The bioavailability of simeprevir is increased when administered with food, resulting in a 60% to70% increase in AUC and a 1 to 1.5 hour delay in absorption. It is therefore recommended to be administered with food. Accumulation of simeprevir occurs after repeated dosing and steady-state concentrations are achieved after 7 days of therapy (106). Elimination of simeprevir is via the biliary system with >91% of drug recovered in the feces. Terminal elimination half-life is 41 hours in HCV infected patients. Renal elimination is negligible and there are no recommended dose adjustments at any level of renal insufficiency (107).

Simeprevir relies heavily on the CYP 3A4 enzyme for metabolism. Therefore, use of moderate to strong CYP3A4 inhibitors and inducers in combination with simeprevir is not recommended due to clinically significant variations in simeprevir exposure. Additionally, simeprevir mildly inhibits intestinal CYP3A4 (no effect on hepatic CYP3A4), CYP1A2, OATP1B1/3, and P-gp activity and may result in increased exposure of drugs that are metabolized or transported by these enzymes (107).

Adverse Effects

Adverse effects of simeprevir that have been reported in >10% of study participants when used in combination with sofosbuvir include fatigue, headache, nausea, insomnia, pruritus, dizziness, and diarrhea. Elevations in direct and indirect bilirubin have been seen early after initiation with simeprevir therapy with peak bilirubin levels seen after 2 weeks of treatment, although these are usually <2.5 × the upper limit of normal (ULN) and resolve rapidly after treatment discontinuation. Close monitoring of hepatic function is warranted as cases of hepatic decompensation, hepatic failure, and death have been reported in postmarketing analyses with the majority of these cases in patients with advanced or decompensated cirrhosis. Due to these risks, simeprevir is not recommended in Child-Pugh B or C, histologic classification of hepatitis C and treatment discontinuation should be considered when elevated bilirubin is accompanied by elevations in liver transaminase or other signs of hepatic decompensation (107).

Rash and photosensitivity reactions have been reported to occur in 28% of patients treated with simeprevir with 56% of these reactions occurring in the first 4 weeks of treatment. Most of these reactions were mild to moderate in severity. Although simeprevir has a sulfonamide moiety, patients with a history of a sulfa allergy have not experienced an increased incidence of rash or photosensitivity in clinical trials of simeprevir. To decrease the incidence of photosensitivity reactions, all patients taking simeprevir should be instructed to use sun protection measures (107).

Resistance

Variants harboring Q80K/R, S122R, R155K, and D168A/V/E substitutions were all found to decrease susceptibility to simeprevir *in vitro*. The most common simeprevir-associated RAVs to emerge in patients who failed to achieve SVR in clinical trials were Q80R, R155K, and D168E/V/X. Efficacy of simeprevir is significantly reduced in patients with HCV genotype 1a with a baseline Q80K polymorphism; screening all genotype 1a patients for this polymorphism before treatment initiation is strongly recommended. Cross-resistance may occur for other NS3/4A inhibitors; patients who have failed treatment with protease inhibitors should not receive simeprevir (107).

Clinical Applications

Simeprevir has been studied in clinical trials among various combination regimens for the treatment of chronic HCV genotype 1. In placebo-controlled randomized phase 3 trials including treatment-naïve, genotype 1 HCV-infected patients, simeprevir in combination with peg-IFN and ribavirin achieved higher SVR 12 rates compared to peg-IFN and ribavirin (80% versus 50%) ($P < 0.0001$) (108, 109). In genotype 1 HCV infected patients who relapsed after previous treatment, simeprevir in combination with peg-IFN and ribavirin achieved SVR 12 rates of 79.2% compared to 36.1% with peg-IFN and ribavirin ($P = 0.001$) (110).

Simeprevir has also been studied in combination with sofosbuvir in genotype 1 HCV infected patients. In previous nonresponders with fibrosis stage ranging from F0 to F2, simeprevir in combination with sofosbuvir with or without ribavirin for 12 to 24 weeks achieved overall SVR12 rates of 90%. In a cohort including treatment-naïve patients as well as previous nonresponders with fibrosis stage ranging from F3 to F4, simeprevir in combination with sofosbuvir with or without ribavirin for 12 to 24 weeks achieved overall SVR 12 rates of 94% (111). In all the subgroups, neither addition of ribavirin nor extension of the treatment duration to 24 weeks had a clinically significant impact on SVR 12 rates. SVR12 rates with simeprevir and sofosbuvir combination therapy are high even in patients with the Q80K polymorphis (111). Simeprevir in combination with sofosbuvir is more effective at achieving SVR 12 than peg-IFN, ribavirin, and sofosbuvir in patients with HCV genotype 1a related Child-Pugh A cirrhosis (93% versus 75%, $P = 0.02$) (112).

Simeprevir in combination with peg-IFN and ribavirin has also been studied in patients coinfected with HCV genotype 1 and HIV-1, yielding SVR 12 rates of 57.1%, 70%, 86.7%, and 79.2% in prior null responders, prior partial responders, prior relapsers, and treatment-naïve patients, respectively (113). Post–liver transplant patients with histological evidence of HCV genotype 1 recurrence were treated with simeprevir in combination with sofosbuvir with or without ribavirin and achieved SVR 12 rates of 90%, although these rates were slightly lower in patients with Metavir F3 to F4 fibrosis (114).

Paritaprevir

Spectrum of Activity

Paritaprevir (2*R*,6*S*,12*Z*,13a*S*,14a*R*,16a*S*)-*N*-(Cyclopropylsulfonyl)-6-{[(5-methyl-2-pyrazinyl)carbonyl]amino}-5,16-dioxo-2-(6-phenanthridinyloxy)-1,2,3,6,7,8,9,10,11,13a,14,15,16,16a-tetradecahydrocyclopropa[*e*]pyrrolo [1,2-*a*][1,4]diazacyclopentadecine-14a(5*H*)-carboxamide) is an NS3/4a inhibitor with EC_{50} of 1.0, 0.21, 5.3, and 0.09 nM against HCV genotypes 1a, 1b, 2a, and 4a, respectively. EC_{50} against genotype 3 is diminished (19 nM), likely related to the presence of the D168Q variant in the NS3 gene (115). It is currently commercially available only in coformulation with ritonavir and ombitasvir and should only be used for treatment of HCV genotype 1a or 1b (in combination with dasabuvir) or genotype 4.

Pharmacokinetics

In healthy volunteers, paritaprevir achieves peak concentration at about 4 hours after administration and has a plasma elimination $t_{1/2}$ of 5.8 hours (85). Median steady state $AUC_{0–24}$ is 2,220 ng × hr/ml. Administration with a fatty meal leads to a 220% increase in AUC compared to fasting conditions (116). It is 97% to 99% protein bound. Approximately 90% is excreted through the biliary tract, and there is only minimal urinary elimination. In individuals with Child-Pugh class C cirrhosis, AUC of paritaprevir was 950% higher than normal controls; therefore, its use is contraindicated in this population. However, paritaprevir may be administered to individuals with Child-Pugh class A or B cirrhosis, as the increase in levels was more moderate in these groups (85).

Paritaprevir is primarily metabolized by CYP3A. It requires coadministration with ritonavir, a potent inhibitor of CYP3A, which allows for once-daily dosing of paritaprevir at a lower dose. Paritaprevir is both an inhibitor and substrate of organic anion transporting polypeptide (OATP) 1B1/B3 and an inhibitor and substrate of P-glycoprotein (P-gp) and BCRP (88). There are many clinically significant drug interactions associated with paritaprevir and ritonavir, primarily caused by the inhibition of CYP3A by ritonavir.

Coadministration with strong CYP3A inducers, such as carbamazepine, phenytoin, or rifampin is contraindicated. Paritaprevir and ritonavir will increase levels of HMG-CoA reductase inhibitors and increase risk of associated adverse events; coadministration of lovastatin and simvastatin are contraindicated. If rosuvastatin is used, it cannot be dosed at more than 10 mg per day. Ergot derivatives are contraindicated with paritaprevir and ritonavir, as are alpha-1 adrenergic antagonists. In clinical trials, coadministration of coformulated ombitasvir/paritaprevir/ritonavir and dasabuvir with products containing ethinyl estradiol was associated with abnormalities in liver function tests, and is therefore contraindicated. Sildenafil coadministration is contraindicated as its levels will be significantly increased (117). Administration with ketoconazole is associated with nearly 100% increase in paritaprevir AUC; if ketoconazole or itraconazole are to be coadministered, a reduced dose of paritaprevir should be given. Voriconazole is also contraindicated with paritaprevir and ritonavir, due to significant reduction in voriconazole levels (118). Fluticasone coadministration with ritonavir has been associated with Cushing's syndrome, and alternatives should be considered (119). Care must also be taken with coadministration of antiarrhythmics and immunosuppressants with therapeutic drug levels monitored whenever possible (120).

With regard to antiretrovirals, paritaprevir has only been studied with atazanavir, raltegravir, and tenofovir/emtricitabine. If coadministered with coformulated ombitasvir/paritaprevir/ritonavir, atazanavir must be given without the usual extra dose of 100 mg of ritonavir. Coadministration with atazanavir is associated with indirect hyperbilirubinemia (121). Rilpivirine, efavirenz, and coformulated lopinavir/ritonavir cannot be given with paritaprevir. For a full account of drug-drug interactions associated with coformulated ombitasvir and coadministered dasabuvir and ribavirin, please refer to the sections pertaining to these medications.

Adverse Effects

There are no data available on the adverse effects of paritaprevir alone, as it was coformulated with ritonavir and ombitasvir early in drug development. For a detailed review of adverse events associated with this coformulation, please refer to the section on ombitasvir.

Resistance

The most common RAV seen in the NS3 protein in patients experiencing virologic failure after receiving paritaprevir is the D168V, which is associated with a 96-fold change in EC_{50} compared to wild-type virus (89, 115). Other common RAVs associated with high-level resistance include Y56H and R155K (89).

Clinical Applications

Paritaprevir is only commercially available coformulated with ombitasvir and ritonavir. For a description of clinical studies involving paritaprevir, please refer to the section on ombitasvir.

Grazoprevir

Spectrum of Activity

Grazoprevir (1a*R*,5*S*,8*S*,10*R*,22a*R*)-5-*tert*-butyl-*N*-{(1*R*,2*S*)-1-[(cyclopropylsulfonyl)carbamoyl]-2-ethenylcyclopropyl}-14-methoxy-3,6-dioxo-1,1a,3,4,5,6,9,10,18,19,20,21,22,22a-tetradecahydro-8*H*-7,10-methanocyclopropa[18,19][1,10,3,6]dioxadiazacyclononadecino[11,12-*b*]quinoxaline-8-carboxamide) has pan-genotypic activity, and has a sub- to low-nM EC_{50} value *in vitro* against genotypes 1a, 1b, and 2 (122).

Mechanism of Action

Grazoprevir is a macrocyclic NS3A/4 protease inhibitor that has a novel mechanism of noncovalent binding to the S2 site of the enzyme that prevents it from interacting with the R155 or D168 residues at the S2 subsite, as do most other macrocytic compounds. It is this property that is believed to allow grazoprevir activity against variants with RAVs at these sites (122–124).

Pharmacology

Grazoprevir peak levels of 165 ng/ml are achieved an average of 3 hours after administration of medication (125, 126). Plasma elimination $t_{1/2}$ is 35 to 39 hours in subjects without renal or hepatic impairment (125, 126). In individuals with severe renal impairment, although grazoprevir exposure is increased somewhat, renal clearance is minimal. Therefore, it is believed to be safe to administer to patients with end-stage renal disease (125). In subjects with Child-Pugh B cirrhosis, AUC and C_{max} are both increased approximately 5-fold compared to healthy controls (126).

Grazoprevir is a substrate of CYP3A4, P-gp, and OATP1B1. It is also a weak CYP3A4 inhibitor and an inhibitor of CYP2C8 and UGT1A1. As a substrate of CYP3A4, levels of grazoprevir are significantly increased when it is coadministered with ritonavir-boosted HIV protease inhibitors; coadministration is, therefore, contraindicated. Although it has not been studied with cobicistat, coadministration is not recommended given the same concern. Although efavirenz coadministration is not recommended, coadministration of fixed-dose combination grazoprevir/elbasvir with rilpivirine did not result in clinically significant changes in levels of any of the three medications, so coadministration is allowable (127). Coadministration with raltegravir or dolutegravir is considered safe (128, 129). Grazoprevir may be safely coadministered with pitavastatin and pravastatin; however, both atorvastatin and rosuvastatin should be avoided (130).

Adverse Effects

There are limited data available on the adverse effects specifically attributable to grazoprevir, as all phase 2 and 3 studies have included coformulated elbasvir. For details of the adverse effects associated with the grazoprevir/elbasivr fixed-dose combination, please refer to the section on elbasvir.

Resistance

Grazoprevir demonstrated a sub-nM potency *in vitro* against the virus with R155K and D168V/Y substitutions (122), which are commonly associated with treatment failure of first-generation NS3/4A inhibitors. EC_{50}s against strains with the A156T substitution were notably higher.

In phase 2 and 3 studies, SVR12 rates were similar in patients with baseline NS3/4A RAVs compared to those without RAVs. The most common NS3 RAVs seen in patients not achieving SVR12 were Y56H, Q80K, A156T, and D168A (131, 132). In a study of grazoprevir/elbasvir in patients who had previously failed treatment that had included a first-generation NS3/4A inhibitor, all three patients who failed developed the emergent R156T substitution, in addition to the reemergence of other baseline RAVs (133).

Clinical Applications

Grazoprevir is in clinical development exclusively in fixed-dose combination with elbasvir, an NS5A inhibitor, so all clinical trial data of grazoprevir pertains to this coformulation. For further detail, please refer to the section on elbasvir.

Asunaprevir

Spectrum of Activity

Asunaprevir (tert-Butyl{(2S)-1-[(2S,4R)-4-({7-chloro-4-methoxyisoquinolin-1-yl}oxy)-2-({(1R,2S)-1-[(cyclopropanesulfonyl)carbamoyl]-2-ethenylcyclopropyl}carbamoyl) pyrrolindin-1-yl]-3,3-dimethyl-1-oxobutan-2-yl}carbamate; Sunvepra) is an NS3/4A inhibitor with activity against HCV genotypes 1a, 1b, and 2 with EC_{50} values of 4, 1.2, and 230 nM, respectively. Antiviral activity against genotype 1 is greater than genotype 2 (134).

Pharmacology

Asunaprevir's C_{max} of 123 ng/ml is achieved in 2.5 hours. Absorption parameters were not significantly affected by food; therefore, asunaprevir can be taken without regard to meals. Steady-state concentrations are achieved after 5 days and total drug exposure is 804.1 ng•h/ml. The primary route of metabolism is through the CYP3A pathway and the majority of drug is eliminated via the biliary route. Asunaprevir accumulates in hepatic dysfunction and is not to be used in patients with Child-Pugh B or C cirrhosis or in patients with decompensated cirrhosis (135–137).

Asunaprevir is a substrate of CYP3A and thus concomitant administration of strong inducers or inhibitors of CYP3A is contraindicated. Asunaprevir is also a substrate of P-gp and OATP 1B1 and 2B1, which are involved in the distribution of asunaprevir to the liver. Strong inhibitors of these enzymes may increase plasma concentrations of asunaprevir while decreasing liver distribution and therapeutic efficacy. Asunaprevir induces CYP3A and inhibits CYP2D6, OATP 1B1, 1B3, and 2B1 and P-gp and may alter exposure of drugs metabolized by these pathways (134, 135).

Adverse Reactions

Elevations in aspartate aminotransferase (AST), ALT, and bilirubin can occur with asunaprevir. It is recommended to monitor liver function tests every 2 weeks for the first 12 weeks of therapy and every 4 weeks thereafter. Treatment should be discontinued permanently if ALT values rise above 10 times the ULN. Other adverse reactions occurring in more than 5% of patients in clinical trials include headache, pyrexia, eosinophilia, nasopharyngitis, and diarrhea (138, 139).

Resistance

Asunaprevir resistant substitutions may occur at the D168 active site and result in EC_{50} values 16- to 280-fold that of the wild-type strain. Cross-resistance to other NS3/4A inhibitors is a concern (140).

Clinical Applications

Although asunaprevir is not approved in the United States, it is widely used internationally as part of combination regimens to treat chronic HCV genotypes 1 and 4. Although early clinical trials of asunaprevir with peg-IFN and ribavirin yielded SVR24 rates ranging from 64% to 89%, these regimens are no longer recommended (141). Recommended regimens include asunaprevir in combination with daclatasvir as well as in combination with daclatasvir and beclabuvir. A fixed-dose combination of asunaprevir, daclatasvir, and beclabuvir is available internationally and clinical applications of this regimen are described in the beclabuvir section.

Several clinical trials performed in Japan evaluated asunaprevir in combination with daclatasvir for genotype 1b HCV. In treatment-naïve patients, the combination of asunaprevir and daclatasvir for 12 weeks achieved SVR12 rates of 90% (142). In previous nonresponders, 24 weeks of this regimen induced SVR12 in 80% to 90% of patients. In IFN-ineligible or intolerant patients, SVR12 rates ranged from 63% to 87% with 24 weeks of therapy (142–144).

Asunaprevir will continue to be an option for combination therapy regimens against HCV genotypes 1 and 4 internationally; however, there are no plans to seek regulatory approval in the United States for this agent.

Vedroprevir

Vedroprevir (1R, 2R-1-[[(2S, 4R)-1-[(2S)-2-[[1R,5S)-3bicyclo[3.1.0]hexanyl]oxycarbonylamino]-3,3-dimethylbutanoyl]-4-[8-chloro-7-(2-morpholin-4-ylethoxy0-2-[2-9propan-2-ylamino)-1,3-thiazol-4-yl]quinolin-4-yl]oxypyrrolidine-2-carbonyl] amino]-2-ethylcyclopropane-1carboxylic acid) is a selective NS3 inhibitor currently in clinical trials for the treatment of genotype 1 HCV. In replicon studies, the EC_{50} was 13 nM in genotype 1a cell lines and was 5.4 nM in genotype 1b cell lines (145). Vedroprevir monotherapy is associated with substitutions at positions 155 and 168 that conferred a >152-fold change in EC_{50} (146). In a phase 2 study of patients given vedroprevir for 6 weeks in combination with sofosbuvir and ledipasvir, 19 (95%) reached SVR12 (101). In a study of this regimen in patients with F3 fibrosis or cirrhosis, the SVR12 rate was 76% (147).

GS-9857

GS-9857 is an NS3/4A inhibitor with pan-genotypic activity. In preclinical studies, it has shown activity against many NS3 RAVs selected by other NS3/4A inhibitors, suggesting a possible role in NS3/4A treatment-experienced patients (148). In a phase 2 study of 6 weeks of treatment with GS-9857 in a fixed-dose combination with sofosbuvir and velpatasvir in genotype 1 patients, SVR12 rates were 93%, 87%, and 67% among treatment-naïve noncirrhotic patients, treatment-naïve cirrhotic patients, and patients

with treatment failure on prior DAA-based regimens, respectively (149). Phase 2 studies of the fixed-dose combination in other genotypes and to evaluate different lengths of treatment are ongoing.

Glecaprevir (ABT-493)

Glecaprevir (ABT-493, Cyclopropanecarboxamide, N-((((1R,2R)-2-((4,4-difluoro-4-(3-hydroxy-2-quinoxalinyl)-2-buten-1-yl)oxy)cyclopentyl)oxy)carbonyl)-3-methyl-L-valyl-(4R)-4-hydroxy-L-prolyl-1-amino-2-(difluoromethyl)-N-((1-methylcyclopropyl)sulfonyl)-, cyclic (1->2)-ether, (1R,2R)) is a pan-genotypic NS3/4A inhibitor being developed in combination with pibrentasvir, a pan-genotypic NS5A inhibitor. Current studies are evaluating various doses of these two drugs in combination with or without ribavirin in patients with genotypes 1, 2, and 3. In noncirrhotic patients with genotype 1 HCV, the combination of glecaprevir and pibrentasvir for 8 weeks achieved SVR12 in 97% of patients, and there were no virologic failures (150). In noncirrhotic patients with genotypes 2 and 3 and who were either treatment-naïve or -experienced, glecaprevir in combination with pibrentasvir for 12 weeks achieved 96% to 100% SVR12 in genotype 2 patients and 83% to 94% SVR12 in genotype 3 patients (151, 152). Adverse effects associated with this regimen include fatigue, nausea, diarrhea, and headache, and are typically mild; however, two patients discontinued therapy due to adverse effects in one trial (150–152). This regimen also has a high barrier for resistance and is active against common HCV harboring NS3/4A an NS5A RAVs (153).

Direct-Acting Agents: NS5A Inhibitors

The NS5A protein plays an essential role in HCV replication, although the specific function of this protein remains unclear. Regardless, NS5A inhibitors have been shown to have high potency against HCV and are generally active against multiple genotypes. The first compounds with activity against NS5A were found by high throughput screening with the genotype 1b replicon. A variety of strategies to refine early compounds were employed to improve the bioavailability, broaden activity to other genotypes, and produce more favorable pharmacokinetics. As a class, these agents tend to have a low barrier to the development of resistance, and as such, are only administered in combination with other HCV DAAs. Characteristics of specific agents that have regulatory approval or are in late stages of clinical development are described below. Medications in earlier clinical development are summarized in Table 3. Figure 3c shows the chemical structures of NS5A inhibitors commonly used in clinical practice.

Daclatasvir

Spectrum of Activity

Daclatasvir (carbamic acid,N,N′-[[1,1′-biphenyl]-4,4′-diylbis[1H-imidazole-5,2-diyl-(2S)-2,1-pyrrolidinediyl[(1S)-1-(1-methylethyl)-2-oxo-2,1-ethanediyl]]]bis-,C,C′-dimethyl ester, hydrochloride (1:2); Daklinza) has antiviral activity against genotypes 1 through 5. Median EC_{50} values for genotypes 1a, 1b, 2, 3, 4, and 5 are 0.008 nM, 0.002 nM, 16 nM, 0.2 nM, 0.025 nM, and 0.004 nM, respectively (154). Daclatasvir was the first NS5A inhibitor to be reported to have antiviral activity in patients with HCV. The development of a dimeric molecule was noted to be an important step in improving the potency of the molecule. On the basis of resistance data, it is believed to bind to the N terminus of the NS5A protein, where the protein is anchored to the endoplasmic reticulum membrane (155).

Pharmacology

Peak plasma concentrations of daclatasvir are observed within 2 hours of administration. The oral bioavailability is 67% yielding mean AUC_{0-24h} values of 10,973 ng•h/ml (156). It is metabolized through the CYP3A4 pathway; however, >97% of drug-related compound measured in plasma is the parent compound. Primary route of elimination is biliary with 88% recovered in feces. The plasma elimination $t_{1/2}$ of daclatasvir is approximately 12 to 15 hours. Pharmacokinetics are not altered in renal or hepatic impairment (157, 158).

Daclatasvir is predominantly metabolized by CYP3A4 and has clinically significant drug interactions with CYP3A4 inducers and inhibitors. When administering daclatasvir with strong CYP3A4 inhibitors, the dose of daclatasvir should be decreased from 60 mg to 30 mg. Administering strong inducers of CYP3A4 (e.g., rifampin, phenytoin, and carbamazepine) with daclatasvir is contraindicated due to risk of lower drug exposure, loss of efficacy, and development of resistance. However, moderate inducers of CYP3A4 can be coadministered with daclatasvir if the dose of daclatasvir is increased to 90 mg (159).

Daclatasvir inhibits P-gp, OATP1B1 and 1B3, and BCRP and can increase the exposure of medications that rely on these pathways for metabolism such as digoxin and dabigatran.

Adverse Effects

The most common adverse effects observed in clinical trials were headache and fatigue, which occurred in greater than 10% of patients. All adverse reactions were mild to moderate in severity and did not lead to treatment discontinuation (163–167).

Resistance

HCV variants with reduced susceptibility to daclatasvir have been identified in cell culture and in clinical studies and occur frequently in patients who experience virologic failure after treatment with daclatasvir. In one phase 3 study, every patient who experienced virologic failure harbored a virus with an NS5A resistance–associated substitution, including A30K/S, L31I, and S62A/L/P/T, with the most common substitution identified at the time of virologic failure being Y93H (160). Treatment emergent resistance–associated substitutions persist at detectable levels for more than 1 year. While baseline polymorphisms are not always associated with decreased efficacy, a baseline Y93H variant is associated with decreased SVR rates (54% versus 92% in those without). Cross-resistance to other NS5A inhibitors is expected; however, effect on other direct-acting antiviral classes is unlikely.

Clinical Applications

Daclatasvir has been studied in clinical trials as part of a variety of regimens against multiple HCV genotypes. In genotype 1 patients, daclatasvir in combination with sofosbuvir given for 12 weeks achieves SVR12 rates of 98% regardless of genotype subtype (1a 98% versus 1b 100%) and previous treatment (treatment-naïve 98% versus prior treatment failure 98%). Neither extending the duration of therapy to 24 weeks nor the addition of ribavirin made an impact on rates of SVR 12 in genotype 1 patients (161). The combination of daclatasvir and sofosbuvir for 24 weeks

achieved SVR12 rates of 92% in untreated genotype 2 patients and of 89% in untreated genotype 3 patients. The addition of ribavirin to the regimen did not improve SVR12 rates in patients with genotype 2 or 3 (162). When the duration of therapy was decreased to 12 weeks in genotype 3 patients, the SVR 12 rates were 90% in treatment-naïve patients and 86% in treatment-experienced patients. Decreasing the length of treatment to 8 weeks is associated with lower SVR12 rates (76%, genotypes 1 to 4), although this may be considered in patients with HCV RNA levels less than 2 million IU/ml (100% SVR12) (163). Subjects with cirrhosis and HCV genotype 3 who received 12 weeks of treatment without ribavirin also had considerably lower SVR12 rates (63%) (160).

SVR12 rates were high (>97%) in patients coinfected with HIV, regardless of genotype or prior treatment experience (160). In post–liver transplant patients with severe recurrent HCV treated with daclatasvir and sofosbuvir, with and without ribavirin for 24 weeks, 75% achieved undetectable levels by the end of treatment; however, 25% of patients died during antiviral therapy. Anti-HCV therapy to treat HCV recurrence after liver transplantation should be initiated at early stages before the onset of cholestasis and liver decompensation to obtain the greatest benefit (164).

Ledipasvir

Spectrum of Activity

Ledipasvir (methyl [(2S)-1-{(1R,3S,4S)-3-[5-(9,9-difluoro-7-{2-[(6S)-5-{(2S)-2-[(methoxycarbonyl)amino]-3-methyl-butanoyl}-5-azaspiro[2.4]hept-6-yl]-1H-imidazol-4-yl}-9H-fluoren-2-yl)-1H-benzimidazol-2-yl]-2-azabi cyclo[2.2.1]hept-2-yl}-3-methyl-1-oxo-2-butanyl] carbamate) has antiviral activity against genotypes 1 through 6, but is most active against genotype 1 with EC_{50} values of 0.018 nM for genotype 1a and 0.006 nM for genotype 1b. Ledipasvir is the least active against genotypes 2a, 2b, 3a, and 6e with EC_{50} values of 21 to 249 nM, 16 to 530 nM, 168 nM, and 264 nM, respectively (165). Ledipasvir, unlike most other NS5A inhibitors, was not developed as a dimeric molecule, but rather is asymmetric. The compound was designed specifically for optimization of coformulations with other DAAs, most notably sofosbuvir (166).

Pharmacology

The plasma concentrations of ledipasvir after a single dose of 90 mg peak approximately 4 hours post dose at 116.5 ng/ml and the $AUC_{0–24}$ is 1,320 ng•h/ml. Ledipasvir absorption is not affected by fat content of meals and can be administered without regard to food intake. Pharmacokinetics are not altered by renal or hepatic impairment. Metabolism occurs via a slow oxidative process. It is eliminated primarily as unchanged parent compound in the feces. The terminal plasma $t_{1/2}$ of ledipasvir is 47 hours (167, 168).

Ledipasvir solubility increases as pH decreases (pH of <2.3) and is practically insoluble between pH of 3 to 7.5. Consequently, it requires an acidic environment to be absorbed and must be appropriately separated from acid reducing pharmacologic agents. Administering ledipasvir under basic conditions can result in lack of absorption and therapeutic effect. Ledipasvir is a substrate of the P-gp efflux pump and use with P-gp inducers is not recommended as there is a risk of a decreased therapeutic effect of ledipasvir. However, ledipasvir can be used concomitantly with P-gp inhibitors. Ledipasvir also inhibits P-gp and BCRP and may increase absorption of coadministered substrates of this transporter.

Adverse Effects

Adverse effects related to ledipasvir are usually mild and rarely cause patients to discontinue therapy (≤1%). The most common side effects seen in clinical trials include headache, fatigue, insomnia, and nausea. Adverse events due to ledipasvir are more common in patients treated with more prolonged courses of therapy (162, 169, 170).

Resistance

Multiple NS5A variants, including Y93H/N, Q30R, and L31M, have been associated with ledipasvir resistance both *in vitro* and in clinical trials, either at baseline and/or at time of virologic failure. In phase 3 clinical trials of ledipasvir-containing regimens, 55% of patients with genotype 1a and 88% of patients with genotype 1b had emergent NS5A resistance–associated substitutions at the time of ledipasvir failure. Baseline resistance-associated variants were seen in 16%, 14%, and 18% of study participants in ION-1, ION-2, and ION-3, respectively. However 89% to 96% of these patients still achieved SVR12 (165, 171, 172). NS5A resistance–associated variants observed in clinical trials with ledipasvir may decrease susceptibility to other NS5A inhibitors (165).

Clinical Applications

Ledipasvir is only commercially available as a fixed-dose combination tablet also containing sofosbuvir. In treatment naïve, genotype 1 patients, ledipasvir used in combination with sofosbuvir for 12 weeks achieved SVR12 rates of 99% (169). Treatment duration may be shortened to 8 weeks in selected patients who are noncirrhotic and have a pretreatment HCV RNA level less than 6 million IU/ml (170). In treatment-experienced patients with HCV genotype 1, the ledipasvir and sofosbuvir combination achieved SVR12 rates of 94% when used in a 12-week regimen and 99% in a 24 week regimen. Patients with cirrhosis had significantly higher SVR12 rates when treated for 24 weeks compared to 12 weeks (95% versus 86%, $P = 0.007$) (162).

Ledipasvir and sofosbuvir combination therapy has also been studied in treatment-naïve and treatment-experienced patients with genotypes 4 and 5 with SVR12 rates ranging from 93% to 95% in genotype 4 and 95% in genotype 5 (171). Studies including patients with genotype 3 are being developed.

Combination therapy with ledipasvir and sofosbuvir has also been studied in patients with HCV and HIV coinfection yielding SVR12 rates of 96% in genotype 1 patients and 100% in genotype 4 patients. The incidence of SVR12 was not affected by cirrhosis or history of treatment (172). Post–liver transplant patients have also been treated with ledipasvir and sofosbuvir in combination with ribavirin for 12 and 24 weeks for chronic HCV with genotypes 1 and 4. SVR rates decreased as liver disease became more advanced with SVR rates ranging from 96% to 98% in fibrosis and Child-Pugh A cirrhosis to 60% to 67% in Child-Pugh C cirrhosis (173).

Ombitasvir

Spectrum of Activity

Ombitasvir (Dimethyl ({(2S,5S)-1-[4-(2-methyl-2-propanyl) phenyl]-2,5-pyrrolidinediyl}bis{4,1-phenylenecarbamoyl(2S)-2,1-pyrrolidinediyl[(2S)-3-methyl-1-oxo-1,2-butanediyl]}) biscarbamate) has activity against HCV genotypes 1 through 5 at an EC_{50} range of 1.7 to 19.3 pM and 366 pM against genotype 6a (174). Ombitasvir, which was the first FDA-approved NS5A inhibitor, was developed as a symmetrical,

pyrrolidine-based structure on the basis of previous observations that dimeric forms of the NS5A protein can possess anti-HCV activity and that inhibitors with dimer-like or symmetric structures seemed to have increased potency against HCV (174). Ombitasvir is coformulated with paritaprevir and ritonavir and administered with dasabuvir as part of the Viekira Pak formulation for genotype 1 HCV and without dasabuvir (Technivie) for genotype 4 HCV. Although ombitasvir and paritaprevir were initially developed by different pharmaceutical companies, a unique licensing agreement between the two allowed the medication to move forward as a coformulation after early clinical trials with ombitasvir and dasabuvir alone failed to show adequate efficacy for some patients with genotype 1 HCV (175).

Pharmacokinetics

Ombitasvir, in its coformulation with paritaprevir and ritonavir, achieves a mean peak concentration of 101 ng/ml at 4 to 5 hours after administration. Mean AUC is 82% higher when administered with a moderate fat meal compared with administration while fasting (116). Plasma elimination $t_{1/2}$ is 25.5 to 32 hours (89). Steady state occurs 12 days after initiation of therapy (86). Ombitasvir monotherapy causes a mean reduction of 3.1 $\log_{10}$ IU/ml in HCV viral load in the first 3 days of treatment (89). Ombitasvir is dosed at 25 mg in the currently available fixed-dose combination pill, but efficacy is similar at doses ranging from 5 mg to 200 mg per day (88). Elimination is primarily through the feces with minimal renal excretion. Concentrations are not affected by renal insufficiency. Ombitasvir, in its coformulation with paritaprevir and ritonavir, is contraindicated in patients with severe hepatic impairment (Child-Pugh C) because of accumulation of paritaprevir (85). P450 enzymes play a minimal role in the metabolism of ombitasvir. It is an inhibitor of UGT1A1 and a substrate of p-glycoprotein and BCRP. Carbamazepine can cause a significant decrease in serum levels of ombitasvir, and their coadministration is contraindicated (88). For a full account of drug-drug interactions associated with coformulated ombitasvir/paritaprevir/ritonavir and coadministered dasabuvir and ribavirin, please refer to the sections pertaining to those medications.

Adverse Effects

Ombitasvir has only been studied as monotherapy in small pharmacokinetic studies with few participants, and no adverse effects were reported (89). Ombitasvir has been studied more widely in phase 3 trials in combination with paritaprevir, ritonavir, and dasabuvir, with or without ribavirin, and it is difficult to know which adverse effects reported in these trials, if any, can be attributed directly to ombitasvir. In two placebo-controlled trials of ombitasvir in combination with paritaprevir, ritonavir, dasabuvir, and ribavirin, subjects in the treatment arm reported increased rates of nausea, pruritus, rash, insomnia, and fatigue (176, 177). Fatigue and nausea were less common in phase 3 studies in which subjects received the direct-acting agents in the absence of ribavirin (178, 179). The incidence of skin reactions reported in phase 3 trials ranged from 7% to 24% of participants and occurred less frequently in subjects not treated concomitantly with ribavirin. There were no reports of Stevens Johnson syndrome or erythema multiforme.

Approximately 1% of patients in phase 3 studies developed ALT elevations greater than 5× the upper limit of normal. The incidence of this elevation was greatly increased among women taking ethinyl estradiol (×). Serum total bilirubin elevation of >2× ULN was noted in 15% of subjects on concomitant ribavirin, and rarely required discontinuation of therapy. Thirty-five percent of HIV coinfected subjects treated concomitantly with ribavirin had serum bilirubin rises to >3× ULN on treatment; however, the majority of these subjects were also receiving atazanavir for HIV infection (121). A drop in baseline hemoglobin level below the lower limit of normal was relatively common in subjects receiving coadministered ribavirin but across all phase 3 studies, no subjects receiving the ombitasvir/paritaprevir/ritonavir plus dasabuvir without coadministered ribavirin experienced a hemoglobin drop below 10 g/dl (87, 178, 179).

Resistance

In a genotype 1a replicon system, ombitasvir selects for resistance variants with substitutions M28T/V, Q30R, H58D, and Y93C/H/N in the NS5A protein. These variants are associated with resistance of 58-fold or greater. Two additional substitutions, Q30H and L31M, were associated with lower-level resistance (2- to 3-fold) (89). In a genotype 1b replicon, the most commonly selected resistant variant was Y93H. The addition of other substitutions leads to higher-level (>400-fold) resistance. In the genotype 4a replicon model, L28V was the only RAV selected, and it was associated with 23-fold resistance to ombitasvir. In clinical trials, the most common NS5A RAV to occur in subjects failing treatment with ombitasvir was the Y30E/K/R, followed by the M28A/T/V. Among all subjects who developed NS5A resistance after receiving ombitasvir in phase 2 trials, 100% had persistence of the RAVs 48 weeks after treatment (86).

Clinical Applications

Ombitasvir has only been studied clinically in combination with paritaprevir, ritonavir, and dasabuvir for genotype 1a and b HCV and in combination with paritaprevir and ritonavir for genotype 4 HCV. In noncirrhotic treatment-naïve subjects with genotype 1a, the DAA regimen, when coadministered for 12 weeks with ribavirin, achieved SVR12 rates of 96%. Similar SVR12 rates were seen in treatment-experienced patients without cirrhosis (176, 179). In a separate study, ombitasvir/paritaprevir/ritonavir plus dasabuvir was associated with a lower SVR12 rate (90%) when administered for 12 weeks without ribavirin (179). Therefore, it is recommended that all noncirrhotic individuals with HCV genotype 1a be treated for 12 weeks with coadministration of weight-based ribavirin, without regard to treatment history. In two studies of noncirrhotic subjects with genotype 1b HCV who received 12 weeks of ombitasvir/paritaprevir/ritonavir plus dasabuvir without ribavirin, SVR12 was achieved in 100% of both treatment-experienced (178) and treatment-naïve patients (179), so this group may receive 12 weeks of treatment without ribavirin.

In treatment-naïve cirrhotic patients with genotype 1a HCV, treatment for 12 or 24 weeks with ombitasvir in combination with paritaprevir, ritonavir, dasabuvir, and ribavirin leads to an SVR12 rate of 94%. Among genotype 1a patients with cirrhosis and a prior null response to treatment, 24 weeks of treatment was associated with higher SVR12 rates than 12 weeks of treatment (93% versus 80%) (180). Among the cirrhotic patients with genotype 1b HCV, response rates were high regardless of history of prior treatment response or whether 12 or 24 weeks were given. Therefore, for patients with cirrhosis and genotype 1b HCV, 12 weeks of therapy with coadministered ribavirin may be given regardless of prior treatment history. Patients with HIV coinfection treated with ombitasvir in combination with

paritaprevir, ritonavir, and dasabuvir for 12 weeks also had an SVR12 rate of 94% (121). The regimen has also been associated with a high SVR12 rate (97%) when given for 24 weeks to patients following liver transplantation (181).

In patients with genotype 4 HCV, 100% of patients (treatment-naïve and -experienced) achieved SVR12 with ombitasvir coformulated with paritaprevir and ritonavir and coadministered with ribavirin (87). SVR12 rates were lower without the coadministration of ribavirin.

Elbasvir

Spectrum of Activity

In both preclinical and clinical studies, elbasvir (dimethyl *N*, *N′*-([(6*S*)-6*H*-indolo[1,2-c][1,3]benzoxazine-3,10-diyl]bis{1*H*-imidazole-5,2-diyl-(2*S*)-pyrrolidine-2,1-diyl[(2*S*)-1-oxo-3-methylbutane-1,2-diyl]})biscarbamate) had activity against a range of genotype 1a and 1b strains as well as genotype 3 strains (182, 183). In a replicon assay, the EC_{90} against genotype 1a virus was under 0.02 nM and for genotype 1b virus was under 0.03 nM.

Pharmacology

Elbasvir has a median T_{max} of 2 to 4 hours, C_{max} of 121 mg/ml, and mean plasma elimination $t_{1/2}$ of 19 to 27 hours, making it amenable to once-daily dosing. Steady-state levels are achieved within 5 days of initiation of therapy (182). Less than 0.15% of the dose is eliminated renally, although C_{max} is 66% higher in individuals with severe renal impairment compared to matched healthy controls, and AUC is 86% higher. It is minimally dialyzable (125).

Elbasvir is a substrate of CYP3A4, P-gp, and OATP *in vitro*. It may also be an inhibitor of BRCP (130). Coadministration with efavirenz was associated with a 50% decrease in elbasvir levels (185). Coadministration with ritonavir-boosted HIV protease inhibitors was associated with large increases in elbasvir AUC; atazanavir/ritonavir was associated with a 4.8-fold rise, lopinavir/ritonavir with a 3.7-fold rise, and darunavir-ritonavir with a 1.7-fold rise (186). Coadministration with raltegravir or dolutegravir has no effect on elbasvir levels (128, 185). Elbasvir coadministration with tenofovir also causes slight increases in the AUC of tenofovir (185).

Coadministration with rosuvastatin is not recommended as this combination can result in large increases in rosuvastatin AUC and C_{max}. Pravastatin levels are more modestly affected, so this should be considered if coadministration of an HMG-CoA reductase inhibitor is indicated (130).

Adverse Effects

There are limited data on the adverse effects attributed specifically to elbasvir. In clinical trials of elbasvir in combination with grazoprevir, the most common adverse effects reported were headache, fatigue, and nausea. Across all of the studies, only one serious adverse event, an episode of abdominal pain, was potentially medication related. There have been infrequent reports of late elevations of AST or ALT; in only one instance was this >5× ULN (131, 132, 184).

Resistance

Treatment of a genotype 1a replicon with elbasvir *in vitro* was associated with the development of the Q30D and Y93N substitutions in NS5A (183). L31F, Y93H, and V121I were the main variants seen in a genotype 1b replicon model treated with elbasvir. Changes at locus 93 were particularly associated with high-level elbasvir resistance. In an elbasvir dose-ranging study, the most common variants to develop were M28T, Q30R, L31V, and Y93H in genotype 1a, and the L31V and Y93H variants in subjects with genotype 1b. The majority of these persisted at 2 months posttreatment (183). In clinical trials of elbasvir/grazoprevir, the presence of baseline NS5A RAVs, especially at position 93 and in subjects with higher baseline viral loads, is associated with lower rates of SVR12, especially in subjects with genotype 1a HCV. The most common NS5A RAVs discovered after virologic failure of elbasvir and grazoprevir were M28A/V/T, Q30H/L/R, L31M, and Y93H/N. These occurred more frequently in patients with genotype 1a (131, 132, 184).

Clinical Applications

Elbasvir is being studied exclusively in fixed-dose combination with grazoprevir, an NS3/4A protease inhibitor. In a large, multisite phase 3 study, 92% of patients with genotype 1a achieved SVR12, as did 99% of participants with genotype 1b, 100% of participants with genotype 4, and 80% with genotype 6 (132). A separate study confirmed high rates of SVR12 among previously untreated genotype 1 participants with cirrhosis treated for 12 weeks without coadministered ribavirin (184). Subjects with HIV/HCV coinfection with genotypes 1, 4, and 6, also achieved high rates of SVR12 with 12 weeks of elbasvir/grazoprevir without ribavirin (131, 187). In individuals who had previously been treated for HCV, SVR12 rates were slightly higher in individuals who received at least 16 weeks of elbasvir/grazoprevir plus ribavirin compared to 12 weeks with no ribavirin, particularly in cirrhotics (100% versus 89% to 91%) (184, 188). In patients with genotype 1 who had previously failed therapy that had included an early generation NS3/4A inhibitor, 96% achieved SVR12 when given elbasvir/grazoprevir plus ribavirin for 12 weeks (133). High rates of SVR12 were also reported in people with Child-Pugh B cirrhosis (189). Elbasvir/grazoprevir given without dosage adjustment was found to be safe and effective in a population of patients with end-stage renal disease, including patients on hemodialysis (190).

Velpatasvir

Spectrum of Activity

Velpatasvir (methyl {(2S)-1-[(2S,5S)-2-(9-{2-[(2S,4S)-1-{(2R)-2- [(methoxycarbonyl)amino]-2-phenylacetyl}- 4-(methoxymethyl)pyrrolidin-2-yl]-1H-imidazol-4-yl}-1,11-dihydro[2]benzopyrano[3′,4′:6,7]naphtho[1,2-d]imidazol-2-yl)-5-methylpyrrolidin-1-yl]-3-methyl-1-oxobutan-2-yl}carbamate) has antiviral activity against genotypes 1 through 6. EC_{50} values for genotypes 1a, 1b, 2a, 2b, 3a, 4a, 5a, and 6a are 13 pM, 15 pM, 9 pM, 10 pM, 13 pM, 9 pM, 59 pM, and 7 pM, respectively (191).

Pharmacology

Velpatasvir achieves its C_{max} of 414 ng/ml at 2.3 hours after a 100 mg dose with an overall AUC of 2,745.3 ng•hr/ml. Velpatasvir undergoes minimal metabolism via hydroxylation and methylation, and parent drug represents approximately 99% of systemic exposure (192, 193). Velpatasvir is predominantly eliminated in the feces with <1% of the dose excreted in the urine. Pharmacokinetics were not significantly altered in patients with renal insufficiency and there are no dose adjustments required in mild, moderate, or severe renal impairment. Velpatasvir has a half-life of 15.3 hours (192, 193).

Velpatasvir is a substrate of CYP3A4, CYP2C8, CYP2B6, and P-gp and may be affected by medications that inhibit or induce these enzymes. Velpatasvir is also a substrate of BCRP and OATP. Velpatasvir also inhibits P-gp, BCRP, and OATP and can increase exposure to medications that rely on these pathways for elimination (197).

Adverse Reactions

Velpatasvir is generally well tolerated with the most common adverse events being headache, fatigue, nasopharyngitis, and nausea. Out of greater than 1,000 patients included in phase 3 studies, <1% discontinued treatment due to adverse drug reactions (194–196).

Resistance

Velpatasvir has improved activity against resistant variants selected by first generation HCV NS5A inhibitors such as daclatasvir and ledipasvir (198). In one phase 3 trial of velpatasvir in combination with sofosbuvir, 60% of patients had baseline NS5A RAVs; however, all of these patients reached SVR12 (195). However, in a different phase 3 trial, SVR12 rates were lower (88%) among patients with baseline RAVs, and the Y93H substitution was most strongly associated with virologic failure (195). The addition of ribavirin or extension of the regimen to 24 weeks may increase SVR12 rates in patients with baseline NS5A RAVs (196).

Clinical Applications

Velpatasvir is currently being studied as a fixed-dose combination with sofosbuvir in patients with HCV genotypes 1 through 6. In patients with genotypes 1, 2, 4, 5, and 6, velpatasvir and sofosbuvir for 12 weeks achieved SVR12 rates of 99%. Nineteen percent of subjects in this study had compensated cirrhosis and 32% were treatment experienced, although patients who had been previously treated with NS5B or NS5A inhibitors were excluded. Rates of SVR were not affected by genotype or the presence of cirrhosis (194). In a study of patients with genotypes 1, 2, 3, 4, and 6 with decompensated cirrhosis, 83% achieved SVR12 with 12 weeks of velpatasvir and sofosbuvir; 94% with 12 weeks of velpatasvir, sofosbuvir, and ribavirin; and 86% with 24 weeks of velpatasvir and sofosbuvir. These differences were not statistically significant (196).

In a separate study, patients with genotype 2 had SVR12 of 99% with velpatasvir and sofosbuvir compared to 94% with sofosbuvir and ribavirin ($P = 0.02$). In genotype 3 patients, the SVR12 rate in the velpatasvir plus sofosbuvir group was 95% compared to 80% in those treated with sofosbuvir and ribavirin (P of <0.001). SVR12 rates among patients receiving velpatasvir and sofosbuvir were lower among subjects with cirrhosis (91% versus 97%) and subjects who had experienced previous treatment failure (90% versus 97%) (195).

Pibrentasvir (ABT-530)

Pibrentasvir (ABT-530) (Methyl {(2S,3R)-1-[(2S)-2-{5-[(2R,5R)-1-{3,5-difluoro-4-[4-(4-fluorophenyl)piperidin-1-yl]phenyl}-5-(6-fluoro-2-{(2S)-1-[N-(methoxycarbonyl)-O-methyl-L-threonyl]pyrrolidin-2-yl}-1H-benzimidazol-5-yl)pyrrolidin-2-yl]-6-fluoro-1H-benzimidazol-2-yl}pyrrolidin-1-yl]-3-methoxy-1-oxobutan-2-yl}carbamate) is a pan-genotypic NS5A inhibitor being developed in combination with glecaprevir, a pan-genotypic NS3/4A inhibitor. Phase 2 data suggest that this regimen is highly efficacious with relatively few adverse events and a high barrier to resistance. For more detail of early clinical trials, please refer to the section on glecaprevir.

Ravidasvir (PPI-668)

Ravidasvir (PPI-668, Methyl N-[(2S)-1-{(2S)-2-[5-(6-{2-[(2S)-1-{(2S)-2- [(methoxycarbonyl)amino]-3-methylbutanoyl}pyrrolidin- 2-yl]-1H-imidazol-4-yl}naphthalen-2-yl)-1H-benzimidazol- 2-yl]pyrrolidin-1-yl}-3-methyl-1-oxobutan-2-yl] carbamate) is an NS5A inhibitor with pan-genotypic activity at a sub- to low-nM EC_{50} against all genotypes (199). High rates of SVR12 were noted when given to patients with genotype 1a in combination with faldaprevir and deleobuvir with or without ribavirin, although it is no longer in development with these agents. It is currently being studied in combination with sofosbuvir in patients with genotype 4 infection.

Direct-Acting Agents: Other Mechanisms

Alisporivir

Spectrum of Activity

Alisporivir (3S,6S,9S,12R,15S,18S,21S,24S,27R,30S,33S)-25,30-diethyl-33-[(E,1R,2R)-1-hydroxy-2-methylhex-4-enyl]-1,4,7,10,12,15,19,27,28-nonamethyl-6,9,18-tris(2-methylpropyl)-3,21,24-tri(propan-2-yl)-1,4,7,10,13,16,19,22,25,28,31-undecazacyclotritriacontane-2,5,8,11,14,17,20,23,26,29,32-undecon) has activity against replicons of HCV of all genotypes tested at a sub-nM EC_{50} (200, 201).

Mechanism of Action

Alisporivir is a second-generation, nonimmunosuppressive cyclophilin inhibitor. This class of medications was developed after the observation that cyclosporine A, a cyclophilin inhibitor used as an immunosuppressant after liver transplantation, has activity against HCV *in vitro* and some efficacy in clinical trials when used in combination with IFN (202–204). The mechanism of action of alisporivir remains incompletely understood. Alisporivir binds to cyclophilin A, a host cytosolic protein that interacts with the HCV NS5A protein and may induce NS5A binding to HCV RNA. It may also impact the activity of NS5B, and, as a result, directly impact viral replication. Cyclophilin A also binds IFN regulatory proteins, making immune modulation another potential mechanism of action (205).

Pharmacology

Alisporivir reaches peak levels an average of 2 hours after administration (206). The formulation being studied in clinical trials is a microemulsion, which is believed to lead to increased bioavailability of the compound (207).Terminal $t_{1/2}$ is 100 hours, which allows for once-daily dosing. Steady state is reached by day 14. Alisporivir is excreted through bile. Mean reduction in serum HCV viral load with alisporivir monotherapy is greatest with genotype 3 virus (206). Alisporivir is both a substrate and an inhibitor of CYP3A4 so is expected to have clinically significant interaction with inhibitors, inducers, and substrates of CYP3A4 (207).

Resistance

Based upon data from a replicon model, alisporivir is believed to have a high genetic barrier to resistance, requiring multiple substitutions in the NS5A domain to increase the EC_{50} to clinically significant levels (208). In clinical trials, the most common variant associated with treatment failure is the D320E in the NS5A domain, which is associated with a 3- to 5-fold increase in EC_{50}. D316E/N and R347R/G/W

are also RAVs that have been associated with alisporivir exposure (209, 210).

Clinical Applications

Alisporivir does not have regulatory approval for use in clinical practice. In a phase 3 study of response-guided therapy with coadministered peg-IFN-alpha2a and ribavirin, 69% of participants receiving alisporivir achieved SVR12. However, a partial clinical hold placed on alisporivir midway through the study limited exposure to medication. In the subset of patients who received at least 24 weeks of 400 mg of alisporivir twice daily along with peg-IFN and ribavirin, 90% achieved SVR12 (209). Five cases of pancreatitis, one fatal, were noted in the group that received alisporivir, though similar rates were noted in a control group receiving peg-IFN and ribavirin alone, so it is unclear if this was a direct effect of the alisporivir (208). In a phase 2b study in patients with genotypes 2 and 3, patients were initiated on varying doses of alisporivir with or without ribavirin with a planned treatment course of 24 weeks. Those who did not have rapid virologic response (HCV VL < 25 IU/ml at week 4 of treatment, RVR) were also given IFN for the final 20 weeks of treatment. SVR12 rates ranged from 80% to 85%, slightly higher in participants with genotype 3, although the majority of patients did not reach RVR and were administered interferon (210). A trial of alisporivir with ribavirin without IFN in subjects with genotype 2 or 3 is ongoing. It remains to be seen whether this will be a viable treatment option or if alisporivir might be administered in combination with other DAAs.

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Antirespiratory Virus Agents

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14

This chapter reviews antiviral agents that have been, or are being, developed to treat or prevent respiratory viral infections. Detailed information is provided for approved agents and those in more advanced stages of clinical development. Agents in Phase I human studies or promising approaches that are still in preclinical development are described briefly. The reader is referred to the respective pathogen-specific chapters for full discussions of the viral agents and the diseases they cause.

INFLUENZA VIRUS

Amantadine

Amantadine (1-adamantane amine hydrochloride; Symmetrel), a tricyclic amine with a "birdcage-like" structure (Figs. 1 and 2; Tables 1 and 2), was approved initially in the United States in 1966 for the treatment of influenza A. It is also indicated for Parkinson's disease. Amantadine belongs to the class of drugs known as adamantanes, which also includes rimantadine. Currently the adamantanes should not be used to treat influenza due to widespread resistance among circulating strains, but the class remains important given the lessons learned and the potential susceptibility of future influenza strains. For example, the seasonal oseltamivir-resistant influenza A (H1N1) virus that circulated globally in 2008–2009 retained susceptibility to adamantanes.

Spectrum of Activity

Amantadine is active against influenza A virus strains only. The inhibitory concentrations of susceptible isolates are generally in the range of 0.1 to 0.4 μg/ml. At approximately 100-fold-higher concentrations, *in vitro* inhibitory activity against other viral agents, such as influenza B virus and flaviviruses (1), can be demonstrated, but these concentrations are not achievable in humans at non-toxic doses.

Mechanism of Action

Amantadine predominantly inhibits an early step in influenza A virus replication by interfering with the function of the viral M2 protein (2). In the replication cycle (Fig. 2), the homotetrameric M2 protein acts as a transmembrane ion channel facilitating the acidification of the virion interior; hydrogen ion-mediated dissociation of the matrix protein from the nucleoprotein enables release of viral RNA segments into the cytoplasm (Fig. 3). M2 also modulates the pH of the *trans*-Golgi network during transport of viral hemagglutinin (HA). Thus, amantadine may also act at a later step in the influenza A virus replication cycle by altering HA formation for certain strains (3, 4).

Pharmacokinetics

Amantadine is well absorbed, reaching the maximum serum concentration (C_{max}) in 2 to 4 hours following an oral dose. Concentrations in the range of 0.5 to 0.8 μg/ml are typically achieved with the standard dosage of 100 mg twice daily; levels in nasal secretions approximate those in plasma (5). The primary route of elimination is renal, and over 90% of the drug is excreted unchanged in the urine. The plasma elimination half-life ($t_{1/2}$) is 12 to 18 hours in healthy young adults but is substantially prolonged in the setting of renal insufficiency and in the elderly. Dose adjustments are needed for these groups. Patients over the age of 65 years should receive half of the recommended dosage, i.e., 100 mg per day. Patients with a creatinine clearance (CrCl) less than 50 ml/min should also receive a reduced dose.

Adverse Effects

The major adverse effect of amantadine is neurotoxicity. Generally, central nervous system (CNS) effects are minor and include lightheadedness, restlessness, insomnia, and mild cognitive difficulties. Such side effects may occur in up to one-third of patients. More serious neurotoxic reactions, including tremor, seizure, and coma, typically occur in situations in which amantadine accumulates, as in elderly subjects or those with renal failure. The availability of rimantadine in some countries, including the United States, allows prescribers to avoid the use of amantadine in patients with a history of seizure disorder or psychosis. Minor gastrointestinal side effects are also common. Less common adverse effects, usually seen with prolonged dosing, include livedo reticularis, fluid retention, orthostatic hypotension, and urinary retention, the latter presumably related to the anticholinergic properties of the drug. Amantadine has been found to be teratogenic and thus should be avoided during pregnancy unless the potential benefit outweighs the risk (Pregnancy Category C) (6). It is also excreted into human milk, and therefore, should be avoided in nursing mothers.

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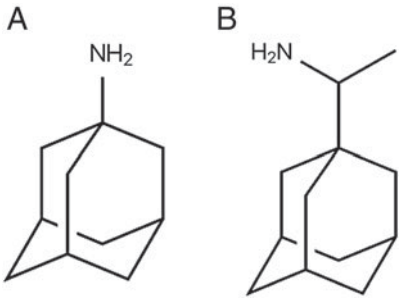

FIGURE 1 Molecular structures of M2 protein inhibitors amantadine (A) and rimantadine (B).

Drug Interactions

The major drug–drug interactions include exposure to agents that potentiate the neurotoxic side effects. Therefore, drugs with antihistaminic or anticholinergic activity should be avoided, particularly in older individuals. Drugs that have been reported to decrease the excretion of amantadine include trimethoprim-sulfamethoxazole, triamterene, and hydrochlorothiazide (7). Amantadine can prolong the cardiac QTc interval and should be used with caution when combined with other QTc-prolonging medications, such as some antipsychotics, fluoroquinolones, and quinidine gluconate.

Resistance

Markedly reduced susceptibility (>100-fold changes) to amantadine and rimantadine is mediated by specific substitutions in the M2 protein of influenza A virus at positions 27, 30, 31, or 34 (8). Resistant isolates have been recovered readily from persons exposed to these drugs. Adamantane-resistant strains can be isolated from 30% of treated subjects within 2 to 5 days, and resistant isolates are transmissible to household or institutional contacts (9). These variants cause typical influenza illness and have no apparent loss of fitness. Although immunocompetent subjects in whom resistant strains emerge generally resolve their illness in the usual time frame when receiving amantadine, the transmission of resistant isolates to contacts abrogates the usefulness of amantadine or rimantadine prophylaxis. Prolonged shedding of resistant variants occurs in immunocompromised hosts.

Since 2003, the prevalence of resistance to adamantanes in circulating influenza A isolates has risen dramatically such that they are not recommended for empiric therapy of acute influenza infection. Adamantane-resistant influenza A virus strains typically possess a substitution in the M2 gene that leads to a serine-to-asparagine substitution at position 31 (S31N) and confers full cross-resistance to rimantadine but does not affect susceptibility to neuraminidase inhibitors (10, 11). Essentially all recently circulating A (H3N2) and A (H1N1) pdm09 viruses are resistant (12, 13). In preclinical models, combinations of amantadine, ribavirin, and oseltamivir have greater therapeutic activity than single agents or dual combinations for influenza A viruses, including adamantine-resistant strains (14), and this triple drug regimen is undergoing a controlled clinical study in outpatients at increased risk for influenza complications.

Clinical Applications

Amantadine is approved for prophylaxis and treatment of susceptible influenza A virus infections, but its use is currently not recommended because of widespread resistance (see Chapter 43 on influenza virus). When this drug is given at the standard dosage of 200 mg/day in adults, the prophylactic efficacy has been consistently reported to be in the range of 70% to 90% for susceptible strains (4, 15, 16). Efficacy has also been shown for postcontact prophylaxis in households, when ill index cases are not treated concurrently, and for outbreak control in closed populations. Amantadine treatment of uncomplicated influenza begun promptly after the onset of symptoms can decrease the duration of fever and

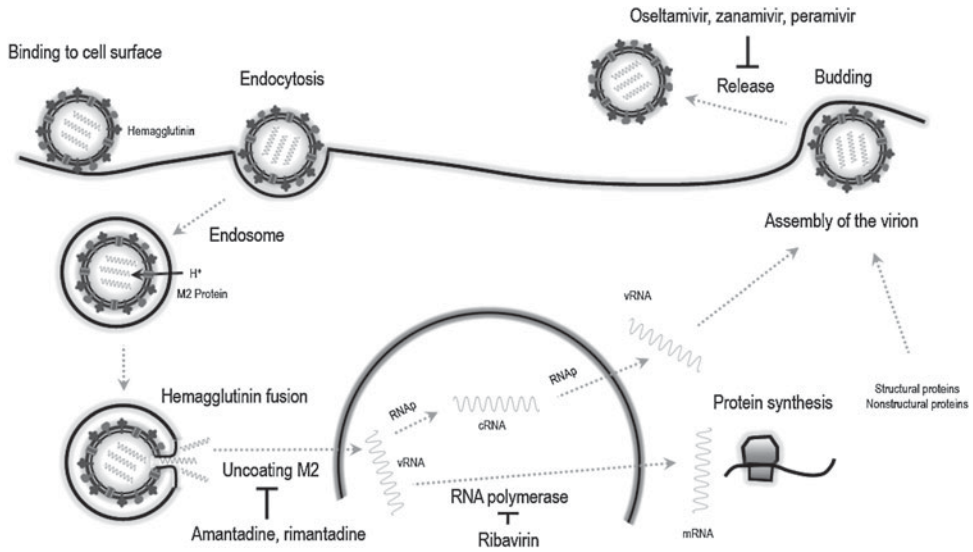

FIGURE 2 Replicative cycle of influenza virus. The influenza virus is shown binding to the surface of an airway epithelial cell via the interaction of hemagglutinin (HA) with sialic acid residues. HA is subsequently cleaved by a protease, and the virus is endocytosed. The M2 protein allows hydrogen ions to enter the virion, promoting the dissociation of ribonuclear protein and release of viral nucleic acids into the cytoplasm (uncoating). Viral RNA replication occurs in the nucleus by the viral RNA polymerase (RNAp) complex. Following replication of viral RNA and synthesis of structural/nonstructural proteins, virions are assembled and released. Sites of action of antiviral compounds are shown by a solid line.

TABLE 1 Antirespiratory virus agents approved in the United States: indications and dosing regimens

Agent	Route	Indications	Adult dosing	Pediatric dosing	Geriatric dosing (≥65 years)	Indications for dose adjustment	
						Renal insufficiency	Hepatic insufficiency
Amantadine	Oral	Influenza A virus Prophylaxis	100 mg p.o. b.i.d. during risk period	Use of amantadine among children aged <1 year has not been evaluated adequately 1–9 years: 5 mg/kg per day in 2 divided doses; maximum daily dose, 150 mg/day ≥10 years and <40 kg: 5 mg/kg per day in two divided doses; maximum daily dose, 200 mg/day ≥10 years and ≥40 kg: 100 mg twice daily	Maximum dose of 100 mg/day for prophylaxis or treatment For certain older persons, dose should be reduced further	CrCl 30–50 mL/minute: administer 200 mg on day 1, then 100 mg/day CrCl 15–29 mL/minute: administer 200 mg on day 1, then 100 mg on alternate days CrCl <15 mL/minute: administer 200 mg every 7 days Hemodialysis: administer 200 mg every 7 days Peritoneal dialysis: no supplemental dose is needed Continuous renal replacement therapy: 100 mg once daily or every other day	No dosage adjustment
		Treatment	100 mg p.o. b.i.d.; discontinue within 3–5 days or within 24–48 hours after symptoms disappear	Refer to prophylaxis dosing	Refer to prophylaxis dosing	Refer to prophylaxis dosing	No dosage adjustment
Rimantadine	Oral	Influenza A virus Prophylaxis	100 mg p.o. b.i.d. during risk period	Use of rimantadine among children aged <1 year has not been evaluated adequately Children 1–9 years: 5 mg/kg per day in one to two divided doses; maximum dose, 150 mg/day Children ≥10 years who weigh <40 kg: 5 mg/kg per day in two divided doses; maximum daily dose, 200 mg/day Children ≥10 years who weigh ≥40 kg and adults, 100 mg twice daily	Maximum dose of 100 mg/day, including elderly nursing home patients	CrCl ≥30 mL/minute: no dosage adjustment necessary CrCl <30 mL/minute: maximum dose, 100 mg daily	Severe dysfunction: maximum, 100 mg daily
		Treatment	100 mg p.o. b.i.d. for 5 days	Refer to prophylaxis dosing	For persons in the community, a reduction in dosage to 100 mg/day should be considered if they experience side effects when taking a dosage of 200 mg/day For nursing home residents, dosage should be reduced to 100 mg/day	Refer to prophylaxis dosing	

(*Continued on next page*)

TABLE 1 Antirespiratory virus agents approved in the United States: indications and dosing regimens (*continued*)

Agent	Route	Indications	Adult dosing	Pediatric dosing	Geriatric dosing (≥65 years)	Indications for dose adjustment	
						Renal insufficiency	Hepatic insufficiency
Oseltamivir	Oral	Influenza A and B viruses Prophylaxis	75 mg p.o. daily for 10 days (up to 6 weeks)	(Off-label) <3 months: not recommended unless situation judged critical because of limited data on use in this age group[a] (Off-label) 3–11 months: 3 mg/kg per dose once daily for 10 days[a] ≤15 kg: 30 mg p.o. once daily for 10 days >15 kg to ≤23 kg: 45 mg p.o. once daily for 10 days >23 kg to ≤40 kg: 60 mg p.o. once dailyfor 10 days >40 kg: 75 mg p.o. once daily for 10 days	No reduction in dosage based on age; usually reduced based on renal impairment	CrCl >60 ml/minute: no dosage adjustment necessary CrCl >30–60 mL/minute: 30 mg once daily CrCl >10–30 mL/minute: 30 mg every other day ESRD not undergoing dialysis: use is not recommended IHD (CrCl ≤10 mL/minute): 30 mg after every other hemodialysis session for recommended prophylaxis duration CAPD: 30 mg once weekly for the recommended prophylaxis duration. Administer immediately after a dialysis exchange	Mild-to-moderate impairment (Child-Pugh score ≤9): no dosage adjustment necessary Severe impairment: no dosage adjustment provided in manufacturer's labeling
		Treatment	75 mg p.o. b.i.d. for 5 days (up to 14 days)	(Off-label) <3 months: 3 mg/kg per dose once daily for 5 days (up to 14 days)[a] (Off-label) 3–11 months: 3 mg/kg per dose once daily for 5 days (up to 14 days)[a] ≤15 kg: 30 mg p.o. twice daily for 5 days (up to 14 days) >15–23 kg: 45 mg p.o. b.i.d. for 5 days (up to 14 days) >23–40 kg: 60 mg p.o. b.i.d. for 5 days (up to 14 days) >40 kg: 75 mg p.o. b.i.d. for 5 days (up to 14 days)		CrCl >60 mL/minute: no dosage adjustment necessary CrCl >30–60 mL/minute: 30 mg twice daily for 5 days CrCl >10 to 30 mL/minute: 30 mg once daily for 5 days ESRD not undergoing dialysis; use is not recommended IHD (CrCl ≤10 mL/minute): 30 mg after every hemodialysis session for 5 days CAPD: 30 mg for one dose to provide a 5-day duration. Administer immediately after a dialysis exchange	

Zanamivir	Inhalational[b]	Influenza A and B viruses Prophylaxis (household) Prophylaxis (community)	Two oral inhalations (10 mg) once daily for 10 days Two oral inhalations (10 mg) once daily for 28 days	Household setting: children ≥5 years and adolescents, two inhalations (10 mg) once daily for 10 days; begin within 36 hours following onset of signs or symptoms of index case Community outbreak: adolescents, two inhalations (10 mg) once daily for 28 days; begin within 5 days of outbreak	No dosage adjustment	No dosage adjustment	No dosage adjustment
		Treatment	Two oral inhalations (10 mg) b.i.d. for 5 days	Children ≥7 years and adolescents: two inhalations (10 mg) twice daily for 5 days. Doses on first day should be separated by at least 2 hours; on subsequent days, doses should be spaced by ∽12 hours. Begin within 2 days of signs or symptoms. Longer treatment may be considered for patients who remain severely ill after 5 days			
Peramivir	Intravenous	Influenza A and B viruses Treatment	300 mg (Japan) or 600 mg (U.S.) intravenously over 15–30 minutes one time only. May consider giving daily for five days in high risk or severely ill hospitalized patients.	28 days to 16 years: 10 mg/kg i.v. over 15–30 minutes one time only	No dosage adjustment	CrCl ≥50 mL/minute: no dosage adjustment necessary CrCl 30–49 mL/minute: 200 mg as a single dose CrCl 10–29 mL/minute: 100 mg as a single dose ESRD IHD: 100 mg as a single dose, administered after dialysis	No dosage adjustment
Ribavirin	Inhalational[c]	RSV	(Off-label) 2 grams (over 2 hours) every 8 hours[d]	Continuous aerosolization; 6 grams administered over 12–18 hours/day for 3–7 days Intermittent aerosolization: 2,000 mg over 2 hours three times daily in nonmechanically ventilated patients for 3–7 days. Use of high-dose therapy in individuals with an endotracheal tube in place is not recommended	No dosage adjustment	No dosage adjustment	No dosage adjustment

[a]Oseltamivir is not approved by the FDA for use in children aged <1 year. An Emergency Use Authorization (EUA) was issued by the FDA on April 28, 2009, but it expired on June 23, 2010.

[b]Intravenous formulation of zanamivir is not approved for use in the United States but may be made available through the Zanamivir Compassionate Use Program for qualifying patients for the treatment of serious influenza illness. For information, contact the GlaxoSmithKline Clinical Support Help Desk at 1-866-341-9160 or gskclinicalsupportHD@gsk.com.

[c]Oral and intravenous formulations are not approved for RSV infection but are used off-label in immunocompromised or severely ill patients. Intravenous ribavirin can be authorized for use as a result of an Emergency Investigational New Drug (EIND) application as investigational treatment for patients with serious viral infections. For information, contact the Valeant Pharmaceutical U.S. medication information at (877) 361-2719.

[d]Aerosol inhalation formulation of ribavirin is not approved by the FDA for use in adults. Off-label use in those patients is often restricted to hematopoietic cell or heart/lung transplant recipients with RSV infection.

p.o., per os; b.i.d., twice per day; CrCl, creatinine clearance; ESRD, end-stage renal disease; IHD, intermittent hemodialysis; CAPD, continuous ambulatory peritoneal dialysis; i.v., intravenous; RSV, respiratory syncytial virus.

TABLE 2 Antirespiratory virus agents approved in the United States: pharmacokinetics, major toxicities, and drug interactions

Agent	Oral bioavailability (%)	Effect of food on AUC	$t_{1/2}$ (h) in adults	Major route of elimination	Major toxicities	Major drug interactions	Comments
Amantadine	90	No	12–18	Over 90% renally excreted unchanged	Neurotoxicity (e.g., lightheadedness, insomnia, seizures less common); nausea; livedo reticularis; orthostatic hypotension; urinary retention	Neurotoxic agents may potentiate amantadine neurotoxic effects; trimethoprim-sulfamethoxazole and triamterene hydrochlorothiazide decrease amantadine excretion. Avoid with QTc-prolonging agents	
Rimantadine	>90	No	24–36	Mainly hepatically metabolized; <25% renally excreted unchanged	Neurotoxicity (less compared to amantadine); nausea, vomiting	None	
Oseltamivir	>75	No	6–10	Hepatic metabolism to active form; renally eliminated	Nausea, vomiting; closely monitor for any neuropsychiatric symptoms in children Monitor for any serious skin and hypersensitivity reactions (e.g., Stevens-Johnson Syndrome, exfoliative dermatitis, or rash) Psychiatric effects (abnormal behavior and hallucinations)	None	Higher doses and longer courses may be used in critically ill patients
Peramivir	N/A	N/A	20	90% renally excreted unchanged	Diarrhea; ALT elevation; glucose elevation; CPK elevation; neutropenia (no difference incidence compared to placebo) Monitor for any serious skin and hypersensitivity reactions (e.g., Stevens-Johnson Syndrome, exfoliative dermatitis, or rash) Psychiatric effects (abnormal behavior and hallucinations)	None	No clinical benefit over oral oseltamivir. Use in patients who cannot tolerate oral medications (e.g., gastric stasis, gastrointestinal bleeding, or vomiting)
Zanamivir	4–17	N/A	2–5	Renal, unchanged	Inhaled: bronchospasm, decreased FEV_1, rash, oropharyngeal edema, headache, cough, nasal symptoms i.v.	None	
Ribavirin	Inhaled: N/A Oral: 64%	N/A	9	Renal and hepatic	Bronchospasm, anemia, neurotoxicity; teratogenic, carcinogenic and mutagenic potential	Azathioprine: levels may be increased of azathioprine's active metabolite Didanosine: toxicity Zidovudine: may increase toxicity of ribavirin	

AUC, area under the curve; $t_{1/2}$, half-life; N/A, not applicable; ALT, alanine transaminase; CPK, creatine phosphokinase; FEV_1, forced expiratory volume; i.v., intravenous.

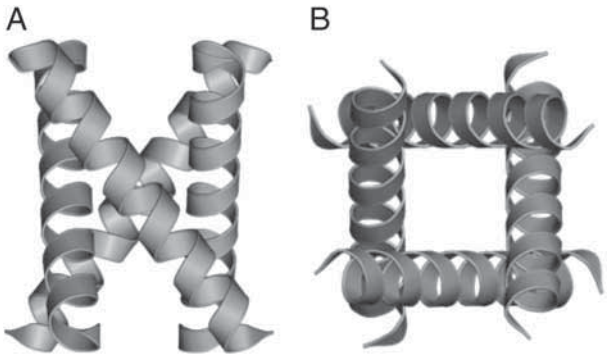

FIGURE 3 Schematic representation of the closed-state structure of the M2 protein channel by solid-state nuclear magnetic resonance (NMR) spectroscopy. Tetrameric assembly is shown from the side (A) and top (B). (Source: Nishimura K et al., *Biochemistry* 2002;**41:**13170–13177.) This image is cited as being in the public domain.

other symptoms by 24 to 48 hours (15). Its usefulness in reducing complications of influenza virus infection or in the management of established influenza A pneumonia is unproven.

Rimantadine

Rimantadine (alpha-methyl-1-adamantane methylamine hydrochloride; Flumadine, Roflual) (Figs. 1 and 2; Tables 1 and 2) is a tricyclic amine that is closely related structurally to amantadine. It was approved for prophylaxis and treatment of influenza in the United States in 1993 (17).

Spectrum of Activity

The activity of rimantadine is exclusively against influenza A viruses at clinically achievable drug concentrations. It is up to 10-fold more active *in vitro* than amantadine, with inhibitory concentrations ranging from 0.01 to 0.1 μg/ml (18). Rimantadine has an inhibitory effect on flaviviruses at higher concentrations *in vitro*, but this has not been shown to correlate with clinical efficacy (19).

Mechanism of Action

The mechanism of action of rimantadine against influenza A virus is identical to that of amantadine.

Pharmacokinetics

Rimantadine, like amantadine, is well absorbed from the gastrointestinal tract, achieving a C_{max} of 0.4 to 0.5 μg/ml after repeated dosing (20). Food does not affect the rate or extent of absorption. However, in contrast to amantadine, rimantadine is concentrated in nasal secretions at a maximal achievable ratio of 1.75:1 (21). The plasma elimination $t_{1/2}$ of rimantadine is 24 to 36 hours, substantially longer than that of amantadine, and only 15% to 20% of an administered dose is excreted unchanged in the urine. The remainder is metabolized hepatically to *ortho-*, *para-*, and *meta*-hydroxy metabolites, which have 100- to 300-fold-less inhibitory activity than the parent compound (22), and then is excreted renally. Older individuals have higher plasma drug levels than young adults when given the same dosage of 200 mg/day. Thus, the dosage should be halved in individuals older than 65 years. In patients with advanced liver or renal disease, the dosage should also be reduced by 50%.

Adverse Effects

The frequency of neurotoxic side effects is lower for rimantadine than amantadine, with treatment cessation rates in clinical trials averaging less than 5%. However, insomnia, minor cognitive difficulties, and, rarely, seizures have occurred. Minor gastrointestinal side effects (e.g., nausea) are common. Rimantadine should be avoided during pregnancy unless the potential benefit outweighs the risk (Pregnancy Category C). It is also excreted into human milk, and, therefore, should be avoided in nursing mothers. As with amantadine, any other drug with neurotoxic potential should be used with caution in rimantadine-treated subjects.

Resistance

The mechanism of resistance to rimantadine is identical to that of amantadine (see above), and complete cross-resistance exists between these two agents. Amantadine- and rimantadine-resistant isolates remain susceptible to the neuraminidase inhibitors.

Clinical Applications

Rimantadine has an efficacy comparable to that of amantadine for either prophylaxis or treatment of uncomplicated, susceptible influenza A virus infections. Rimantadine's improved side effect profile and patient tolerance make it the preferred agent for use in elderly and high-risk persons, when an adamantane is indicated. As with amantadine, rimantadine is currently not recommended for influenza treatment and prevention.

Zanamivir

Zanamivir (5-acetylamino-4-[aminoiminomethylamino]-2, 6-anhydro-3,4,5-trideoxy-d-glycero-d-galacto-non-2-enonic [ol0] acid; Relenza) (Figs. 2 and 4; Tables 1 and 2), is an

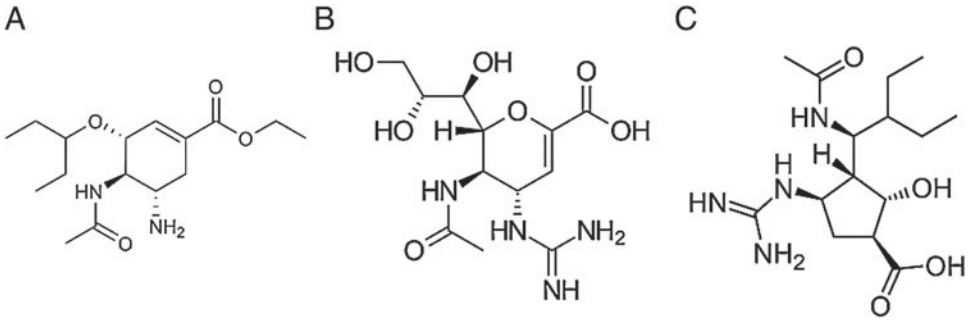

FIGURE 4 Molecular structures of the neuraminidase inhibitors oseltamivir (A), zanamivir (B), and peramivir (C).

influenza virus neuraminidase inhibitor that was approved in the United States for the treatment of influenza A and B virus infections in 1999 and for prophylaxis in 2006.

Spectrum of Activity

Zanamivir was the first approved drug of a class of antiinfluenza agents that potently and selectively reduce the replication of influenza A and B viruses through competitive inhibition of influenza virus neuraminidase activity. The mean zanamivir 50% inhibitory concentration values (IC_{50}s) for influenza virus neuraminidase were found to be 0.76, 1.82, and 2.28 nM for the subtype H1N1 (N1), H3N2 (N2), and B neuraminidase, respectively, in enzyme inhibition assays (23, 24). It has no activity against other viral neuraminidases or influenza C virus.

Mechanism of Action

Zanamivir was specifically designed to bind to the highly conserved active enzyme site of influenza virus neuraminidase (25). Neuraminidase is a surface glycoprotein that catalyzes cleavage of the terminal sialic acid residues attached to glycoproteins and glycolipids that are recognized by viral HA; this action is necessary for release of influenza virions from host cells and for spread of the virus through respiratory mucus. Inhibition of influenza virus neuraminidase by zanamivir and related neuraminidase inhibitors interferes with progeny virus dispersion and reduces infectivity. Zanamivir is a transition state analog of sialic acid that interacts somewhat differently than oseltamivir with viral neuraminidase and consequently has a different spectrum of antiviral activity.

Pharmacokinetics

Zanamivir is currently commercially available only as a dry-powder oral inhalation via a proprietary disk inhaler. Its absolute oral bioavailability is very low (4%), but an intravenous formulation has been developed and used clinically, although not licensed at present for this route of administration. Following a 10-mg inhaled dose of zanamivir, the majority of the drug is deposited in the oropharynx after inhalation, but approximately 13% reaches the lower respiratory tract (26). Less than 15% of the total dose is absorbed with resulting plasma C_{max} of about 40 to 50 ng/ml. The plasma elimination $t_{1/2}$ varies from 2.5 to 5.1 hours, but high sputum concentrations persist up to 12 hours after the dose (27). Because so little is absorbed systemically, the increased drug exposure in individuals with severe renal impairment is not considered clinically relevant; no dosage adjustment is recommended for renal insufficiency or people older than 65 years of age. Elderly patients, especially those with cognitive impairment, may experience difficulty in using the inhaler device.

Intravenously administered zanamivir provides high levels in blood (mean $C_{max} > 39{,}000$ ng/ml with repeated 600-mg doses), is distributed to the respiratory mucosa, and protects against infection and disease after challenge with experimental human influenza A virus (27).

Adverse Effects

Bronchospasm, decreased forced expiratory volume (FEV_1), and decreased peak expiratory flow rates have been seen uncommonly in individuals treated with orally inhaled zanamivir. Severe respiratory distress, particularly in influenza-virus-infected patients with preexisting lung disease, has been associated with hospitalization, and rarely, death. As a result, zanamivir is not recommended in individuals with underlying airway disease, although its use in influenza patients with mild or moderate asthma appears to be safe (28). The commercial lactose-containing formulation of inhaled zanamivir should not be used in mechanically ventilated patients because blockage of filters may occur (29). Adverse events include headache, bronchitis, nausea, vomiting, and diarrhea, all of which occurred in no more than 3% of treated subjects, comparable to the effects of placebo. Allergic reactions, including oropharyngeal edema and skin rashes, occur rarely with zanamivir.

Zanamvir may be administered to pregnant patients because available data suggest that neuraminidase inhibitors are not teratogenic (30–32) and the benefit of treatment outweighs the risk of influenza complications. Gravid rats given 1000 times the human dose of zanamivir delivered offspring with a variety of skeletal alterations; however, at lower doses, there were no malformations, maternal toxicity, or embryotoxicity observed in pregnant rats or rabbits receiving intravenous (i.v.) zanamivir. No increase in adverse pregnancy outcomes or fetal abnormalities has been observed in zanamivir-exposed pregnant women to date (33). Zanamivir is excreted in breast milk in rats, but it is unknown if it is found in human milk.

Drug Interactions

Drug interactions with zanamivir are unlikely. Zanamivir is not an inducer or inhibitor of cytochrome P450 isoenzymes, is not metabolized by the liver, and does not affect drug metabolism in human liver microsomes. Protein binding is low. Inhaled zanamivir does not affect the humoral immune response to injected influenza vaccine but, if administered concurrently, could potentially interfere with immune responses to live-attenuated influenza vaccine.

Resistance

Clinical isolates resistant to zanamivir are very uncommon but have been found in immunocompromised individuals during zanamivir treatment (34). Prolonged treatment of one immunocompromised child with influenza B resulted in the emergence of a dual HA and neuraminidase mutant (position 152) virus with reduced sensitivity (35). An immunosuppressed child with influenza B virus infection had an aspartic acid-198/asparagine (Asp198Asn) substitution, with decreased sensitivity to both oseltamivir and zanamivir (36). In a Japanese study examining 74 children with influenza B virus infection who had received oseltamivir treatment, one had a viral isolate with decreased sensitivity to the neuraminidase inhibitors with a G402S substitution. Among 422 children and adults with untreated influenza virus infection, 7 had isolates with reduced susceptibility to oseltamivir and zanamivir with D198N, I222T, or S250E substitutions (37). During the first 3 years of neuraminidase inhibitor use, from 1999 to 2002, only two ($< 0.1\%$) influenza A (H1N1) viral isolates showed > 10-fold-decreased susceptibility to zanamivir (38). Zanamivir resistance was not observed in other subtypes during the same period (39). Oseltamivir-resistant variants with H275Y in N1 and E119V in N2 neuraminidases remain sensitive to zanamivir; the R292K substitution in N2 or N9 confers reduced zanamivir susceptibility.

Clinical Applications

Inhaled zanamivir is effective in the treatment of uncomplicated influenza A and B virus infections if given within 48 hours of the onset of symptoms (40). Depending in part on time to initiating therapy, zanamivir reduces the duration of

symptoms by 1 to 3 days (40). Early treatment with orally inhaled zanamivir also reduces antibiotic usage in adults and adolescents with influenza (41), but there are insufficient data to establish that zanamivir treatment prevents serious complications of influenza or that it is safe and effective in treating severe influenza. Zanamivir, given at the usual adult dosage of 10 mg twice daily, is safe and effective in children aged 5 to 12 years. Because of a low estimated treatment effect and inadequate inhalation technique in children aged 5 to 7 years, zanamivir treatment is approved by the FDA only for children older than 7 years. Zanamivir prophylaxis is approved for children older than 5 years.

The protective efficacy of zanamivir has been approximately 79% in studies of postexposure prophylaxis (41). Postexposure prophylaxis with zanamivir for amantadine-resistant influenza A virus has also been demonstrated to be safe and efficacious (42). Because zanamivir therapy does not affect antibody responses to injected influenza vaccination, it may safely be given in combination with inactivated vaccine for immediate protection of at-risk persons (43).

Concerns exist about the effectiveness and safety of orally inhaled zanamivir in treating severe influenza, particularly with respect to effective delivery to sites of infection in the lower respiratory tract. Nebulized zanamivir lacking the lactose carrier has received clinical study and use (44, 45). In animal models and *in vivo*, zanamivir is active against influenza A (H5N1) virus, including highly oseltamivir-resistant strains with the H275Y substitution; however, use of zanamivir for A (H5N1) in humans has not been assessed directly (46).

At this time, i.v. zanamivir is only available as an investigational agent in the United States. In an open-label study, i.v. zanamivir was generally well tolerated and appeared to be associated with antiviral effects in critically ill patients (47). A Phase III randomized controlled trial found no significant difference between i.v. zanamivir and oseltamivir in regard to time to clinical response (48). However, i.v. zanamivir is generally well-tolerated and remains of clinical investigative interest, in part because its spectrum of activity includes many oseltamivir-resistant variants. For hospitalized patients infected with oseltamivir-resistant influenza virus (e.g., [H1N1] pdm09 virus), i.v. zanamavir can be obtained under an emergency Investigational New Drug (IND) (Table 1).

Oseltamivir

Oseltamivir [ethyl(3*R*,4*R*,5*S*)-4-acetomido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate; Tamiflu] (Figs. 1 and 3; Tables 1 and 2), is a neuraminidase inhibitor that was approved for treatment of influenza in the United States in 1999 and for prevention of influenza in 2000. It is registered in more than 100 countries and, as of April of 2014, had been used in more than 130 million patients since introduction (49).

Spectrum of Activity

Oseltamivir carboxylate is a potent competitive inhibitor of influenza virus neuraminidase activity. It is specific for influenza A and B virus neuraminidases, and no clinically relevant activity is seen against other viral neuraminidases. Differences in levels of susceptibility to oseltamivir have been demonstrated among influenza A and B viruses, with a lower inhibitory effect on influenza B viruses. In one study, the mean IC_{50}s for influenza A virus clinical isolates in neuraminidase inhibition assays ranged from 0.67 to 1.53 nM, whereas the mean IC_{50}s for influenza B virus clinical isolates varied from 10.01 to 11.53 nM (50). Similarly, in another study, the median IC_{50}s for influenza A virus H1N1 and H3N2 strains were 0.45 and 0.37 nM, respectively, while the median IC_{50} for influenza B virus was 8.50 nM (51). These differences in susceptibility may explain the reduced efficacy of oseltamivir in treating influenza B virus infection in children (52).

Whether oseltamivir has direct immunomodulatory effects is unresolved. One animal model study showed that oseltamivir decreased $CD8^+$ T-cell-mediated immunity to RSV infection, perhaps by blocking endogenous sialidase activity in host lung mononuclear cells, and delayed RSV clearance (53), but the clinical relevance of this finding is unclear.

Mechanism of Action

Oseltamivir is the orally available ethyl ester prodrug of oseltamivir carboxylate, a highly potent influenza virus neuraminidase inhibitor. The carboxylate is a carbocyclic transition state analog of sialic acid that binds to conserved residues within the active sites of influenza A and B virus neuraminidases and dissociates very slowly, effectively inhibiting enzymatic function (53). In preclinical studies, influenza virus neuraminidase has been reported to cleave sialic acid from pulmonary cells to expose receptors for *Streptococcus pneumoniae* adherence. Neuraminidase inhibitors can inhibit this effect and reduce the mortality of secondary bacterial pneumonia in such models (54).

Pharmacokinetics

After oral administration, oseltamivir phosphate (the inactive prodrug) is readily absorbed from the gastrointestinal tract and converted to its active carboxylate metabolite with a bioavailability of at least 75%. The carboxylate is detectable in plasma within 30 min of administration, and reaches a C_{max} of 348 to 551 ng/ml within 3 to 4 hours of a standard 75-mg dose. Administration of food has no significant effect on peak levels and has the advantage of reducing the risk of adverse gastrointestinal effects. In rat models, peak levels of drug within the bronchoalveolar-lining fluid are approximately the same as those in plasma and decline more slowly (55). Oseltamivir and the carboxylate have very low penetration into the CNS with a mean observed C_{max} of 2.4 ng/ml for oseltamivir and 19 ng/ml for oseltamivir carboxyxlate in the CNS (56). Oseltamivir phosphate is rapidly converted by esterases into its active metabolite in the liver. Neither oseltamivir phosphate nor carboxylate interacts with cytochrome P450 mixed-function oxidases or glucuronosyltransferases (57).

The active metabolite is cleared unchanged by glomerular filtration and active tubular secretion in the kidneys, with a $t_{1/2}$ in plasma of 6 to 10 hours in healthy young adults. Because clearance of the drug depends on renal function, individuals with a creatinine clearance less than 60 ml/minute or on hemodialysis (HD) should receive a lower dose (57). Exposure to both oseltamivir and its active metabolite is increased in elderly patients by approximately 25%, but no dose adjustment is necessary for this group because of the drug's relatively benign safety profile. In persons with mild and moderate hepatic disease, the C_{max} of oseltamivir was ≤6% lower in persons with hepatic impairment than that in healthy subjects, whereas the C_{max} of oseltamivir carboxylate was ≤19% lower (58). The mean area under the curve (AUC) of oseltamivir was 33% higher than that in healthy subjects, whereas the mean AUC of the active metabolite was ≤19% lower. Consequently, no dose adjustment of

oseltamivir is needed routinely in persons with hepatic impairment. Polymorphisms affecting oseltamivir pharmacokinetics have been described *in vitro*, but are thought to be of low clinical significance in humans (59).

Adverse Effects

Oseltamivir is generally well tolerated. The most common adverse effects are nausea and vomiting, which occur in 3% to 15% of subjects (57). Abdominal pain, dizziness, and headache were reported to occur in 2% to 20% of subjects, rates comparable to those for patients given placebo (57). Most of these adverse effects resolve after 1 to 2 days of treatment (59). Administration of oseltamivir with food decreases the risk of gastrointestinal side effects. Serious reactions such as aggravation of diabetes, arrhythmia, confusion, seizures, orofacial edema, toxic epidermal necrolysis, and unstable angina occur with a frequency of less than 1%, although a direct causal relationship has not been proven (60).

Serious neuropsychiatric adverse events related to oseltamivir therapy in adolescents were reported in Japan in 2007, prompting Japanese authorities to recommend against prescribing oseltamivir to persons aged 10 to 19 years in that country. In November 2007, the U.S. FDA recommended that revised precautions about serious neuropsychiatric events associated with pediatric oseltamivir use be included in package labeling for the drug. Physicians are advised to monitor patients closely for symptoms of abnormal behavior during oseltamivir treatment and to assess the risks and benefits of continuing therapy in patients who develop these symptoms. In a 2011 publication, the Japanese Ministry for Health, Labour and Welfare issued a preliminary analysis concluding no causal relationship between oseltamivir therapy and occurrence of severe neuropsychiatric events. An American Academy of Pediatrics publication described the incidence of these events to be in the range of 1 in 10,000 to 100,000 treatment courses and with uncertainty regarding whether the effects are from oseltamivir, influenza virus illness, or an interaction of the two (61). Similar types and rates of adverse effects were found in high-risk populations, such as those who have undergone hematopoietic stem cell transplantation and the frail elderly (59).

Pregnant women have a higher risk of complications due to influenza. According to the U.S. CDC, oseltamivir is the preferred treatment agent in pregnant women; treatment should be started as soon as possible after the onset of illness, and prophylaxis should be considered after exposure to influenza (13). In a rabbit model, a maternal dosage of 150 to 500 mg/kg per day resulted in a dose-dependent increase in minor skeletal abnormalities. Studies to date have not found evidence for adverse pregnancy outcomes or teratogenic effects in humans (62–64). Oseltamivir has been detected in breast milk of lactating women, but concentrations were low and unlikely to lead to toxicity in infants (65).

Drug Interactions

Neither oseltamivir nor its carboxylate interacts with cytochrome P450 enzymes. Both compounds have low protein binding. Coadministration of oseltamivir with cimetidine, an inhibitor of cytochrome P450 and competitor for renal tubular excretion, resulted in no effect on the levels of oseltamivir or its metabolite in plasma. Probenecid coadministration results in a 2-fold increase in oseltamivir exposure, but no dose adjustments are required due to the safety margin of oseltamivir. One *in vitro* study suggested that concurrent clopidogrel may reduce the antiviral activity of oseltamivir by inhibiting its hydrolysis to the carboxylate form; the clinical relevance of this effect is unproven (66).

Resistance

Oseltamivir treatment may result in the development of neuraminidase active-site substitutions that decrease neuraminidase binding affinity for oseltamivir carboxylate and confer reduced susceptibility. Various neuraminidase substitutions have been recognized in clinical isolates, most commonly R292K or E119V in A (H3N2) and A (H7N9) viruses, H275Y in A (H1N1) and A (H5N1) viruses, N294S in A (H5N1) virus, and D198N or I222T in B virus (67). The H275Y substitution, found in N1-containing viruses, confers highly reduced susceptibility to oseltamivir (>400-fold higher 50% effective concentration [EC_{50}] in enzyme inhibition assays), but not to zanamivir (68).

Global surveillance results from the Neuraminidase Inhibitor Susceptibility Network during the first 3 years of clinical use of the drug class showed a >10-fold decline in oseltamivir susceptibility in 8 (0.33%) of 2,287 influenza virus clinical isolates. None of these isolates were from persons who had previously received treatment with neuraminidase inhibitors (38). However, during the 2007–2008 season, influenza A (H1N1) viruses with an H275Y substitution emerged and spread globally to replace susceptible strains in the apparent absence of selective drug pressure, in part because of enabling amino acid changes in the neuraminidase that conferred fitness (69). Emergence of resistance in the pandemic A (H1N1) 2009 virus has been much less common, although its prevalence has increased recently and sometimes has been associated with community transmission (70–73). Data from the 2014–2015 influenza season revealed that >98% of A (H1N1) pdm09 strains were susceptible to oseltamivir and peramivir and 100% were susceptible to zanamivir (13).

Emergence of oseltamivir resistance during treatment occurs more frequently in children than in adults (74) and is a particular risk in immunocompromised hosts (75). This may be explained by higher viral titers and longer periods of viral shedding in children (10) and immunocompromised hosts. Most influenza viruses resistant to oseltamivir have mostly remained susceptible to zanamivir and laninamivir, a related investigational neuraminidase inhibitor, but are variably susceptible to peramivir (10).

The H275Y substitution has also emerged in two of eight influenza A (H5N1) virus-infected patients given oseltamivir, both of whom died (76). Emergence of neuraminidase R292K substitution was found in two patients with influenza A (H7N9) virus, resulting in reduced susceptibility to zanamvir and especially oseltamivir though notably, these patients were also receiving corticosteroids (77).

Clinical Applications

Oseltamivir is approved for treatment and prophylaxis of influenza in adults and children (at least 2 weeks of age for treatment and at least 1 year of age for prophylaxis). Oseltamivir is effective for treatment of influenza A and B virus infections in ambulatory adults if given within 36 hours of the onset of symptoms and in children if given within 48 hours or perhaps longer (78). Treatment reduces the risk of complications leading to antibiotic use, including otitis media and perhaps pneumonia in children (79). Oseltamivir treatment of influenza in outpatients is associated with a lower rate of lower respiratory tract complications, necessitating antibiotic therapy and a lower rate of hospitalization (60).

Observational studies in seasonal influenza, pandemic 2009 A (H1N1) pdm09, and avian A (H5N1) infections indicate

that timely oseltamivir therapy can reduce mortality in seriously ill patients. Prompt initiation of oseltamivir reduced risk of mortality, intensive care unit (ICU) admission, and respiratory failure in patients infected with pandemic H1N1 2009 influenza, including pregnant women and immunocompromised hosts both in adults and children (59,80–82). During the 2009 H1N1 pandemic, multiple studies showed that oseltamivir use within 48 hours of illness onset resulted in a shorter duration of viral shedding compared to treatment at a later stage of illness (83–85). However, double-dose oseltamivir therapy does not appear to be more effective than standard dose in hospitalized patients, except possibly in influenza B (86).

For influenza A (H5N1) infection, a survival benefit was observed in persons with oseltamivir treatment compared to those with no treatment (29% to 67% survival rate, versus 0% to 33%). Factors associated with poor prognosis were late initiation of treatment and persistence of viral replication after completion of standard therapy. It is unclear if severe diarrhea and gastroparesis experienced by persons with A (H5N1) infection affect drug absorption and bioavailability (87). A standard 5-day course of oseltamivir is advised. Modified treatment options, such as higher dosages of oseltamivir (150 mg twice daily in adults), prolonged duration (course increased to a total of 10 days), and combination regimen with the adamantanes, should be decided on a case-by-case basis (87).

Postexposure prophylaxis of household contacts with oseltamivir (75 mg once daily) is protective, with an efficacy of 89% (88). Long-term prophylaxis with oseltamivir (75 mg once or twice daily) for 6 weeks during peak influenza virus activity resulted in a protective efficacy of 74% to 82% in adults, depending upon the rate of influenza virus infection in the area, and up to 92% in elderly residents of nursing homes (89, 90). Oseltamivir therapy does not appear to interfere with antibody responses to natural infection or influenza vaccination.

Peramivir

Peramivir (BCX-1812; Rapivab) (Figs. 2 and 4; Tables 1 and 2) is a novel cyclopentane compound that inhibits influenza virus neuraminidase. Its parenteral formulation makes peramivir an important addition to the class in hospitalized patients when oral absorption is compromised. Prior to FDA approval for uncomplicated influenza in adults in the United States in 2014, it was used under emergency authorization in adults and children with serious influenza A (H1N1) pdm09 infection (91–93).

Spectrum of Activity

Peramivir has *in vitro* activity against both influenza A and B viruses (94), including avian influenza A (H5N1) and A (H7N9). Peramivir shares structural similarities with oseltamivir and generally has a comparable *in vitro* spectrum of activity, although with somewhat greater inhibitory effects for influenza B neuraminidases. The median neuraminidase inhibitory activities (IC_{50}s) of peramivir are 0.16 nM (range 0.01–1.77 nM) against A (H1N1), 0.13 nM (range 0.05–11 nM) for A (H3N2), and 0.99 nM (range 0.04–1.77 nM) against influenza B viruses (95).

Mechanism of Action

Peramivir shares the same basic mechanism of action as zanamivir and oseltamivir. The drug has a strong affinity for influenza neuraminidase as well as a slow off rate. This feature and its pharmacokinetic properties enable one-time dosing in uncomplicated infections (96).

Pharmacokinetics

Following a single i.v. dose of 600 mg over 30 minutes, an average C_{max} of 46,800 μg/mL is reached at the end of infusion. The pharmacokinetic parameters indicate a linear relationship between dose and C_{max} and AUC for doses ranging from 100 to 800 mg (96). *In vitro* protein binding is less than 30%.

Peramivir is eliminated primarily by glomerular filtration, with more than 90% of the drug excreted unchanged in the urine. Following a single infusion, plasma elimination of the agent follows a multiexponential decline with a mean residence time of approximately 3 hours; the terminal half-life for the slow phase of elimination is approximately 20 hours (97). The dose should be reduced in patients with a creatinine clearance less than 50 ml/min. Peramivir is removed by dialysis with systemic exposure reduced by 73% to 81% after a 4-hour HD session (97). Peramivir has not been evaluated in patients with liver disease, but is not anticipated to accumulate in such cases because it is not metabolized hepatically (98). Peramivir is not a substrate for CYP enzymes or P-glycoprotein-mediated transport and does not affect glucuronidation. There are no reported drug interactions.

Adverse Effects

In clinical studies, adverse events reported in subjects receiving peramivir included diarrhea, alanine transaminase (ALT) elevation, glucose elevation, creatine phosphokinase (CPK) elevation, and neutropenia. The incidence rates of these effects were similar in study participants receiving placebo. Serious skin and hypersensitivity reactions (Stevens-Johnson syndrome, exfoliative dermatitis, and rash) as well as psychiatric effects (abnormal behavior and hallucinations) have been observed in postmarketing experience in Japan (94). Other serious but rare adverse events possibly associated with peramivir administration include severe thrombocytopenia (99) and exacerbation of myasthenia gravis (100).

No well-controlled studies of peramivir have been conducted in pregnant women, but reproductive studies in rats and rabbits have demonstrated potential developmental toxicities, including abortion, premature delivery, and fetal anomalies, such as reduced renal papilla and dilated ureters (95). Given that, the agent should generally be avoided during pregnancy unless the potential benefit outweighs these risks (Pregnancy Category C). The drug has not been studied in nursing mothers, but data from rats indicate that the agent is excreted in milk at levels below the mother's plasma drug concentration levels (95, 98).

Resistance

Influenza A and B virus isolates with reduced susceptibility to peramivir have been observed in cell culture and *in vivo*. Reduced susceptibility may be conferred by amino acid substitutions (including H275Y in influenza A [H1N1] as well as R292K and N294S in influenza A [H3N2]) in viral neuraminidase (95). These isolates with reduced susceptibility have been identified in community surveillance studies, including patients who have not been exposed to peramivir (95). In 2014, a community cluster of influenza A (H1N1) pdm9 with an H275Y substitution was found to exhibit cross-resistance among oseltamivir and peramivir in 6 patients (101). This resistance substitution has also emerged during i.v. peramivir use in immunocompromised hosts (102).

Clinical Applications

In the United States, peramivir is approved for the treatment of acute uncomplicated influenza in patients 18 years and

older who have been symptomatic for no more than 2 days. In two double-blind, placebo-controlled studies of adults with acute, uncomplicated pneumonia (101, 103) an integrated analysis showed that peramivir offered a reduction in time to alleviation of symptoms compared to placebo (113.2 hours vs. 134.8 hours; unadjusted P=0.047). The duration of fever was also shortened by approximately 24 hours (P=0.004) (103). Despite these results, a subsequent Phase II multicenter, randomized, placebo-controlled study of intramuscular (i.m.) peramivir (104) failed to show a difference in time to alleviation of symptoms between the study drug and placebo (91.1 hours vs. 106.9 hours, respectively, P=0.22) (105). These results have been attributed to a high prevalence of influenza A (H1N1) with the H275Y substitution conferring resistance to peramivir (105).

Peramivir i.v. has also been evaluated in both outpatients and in those hospitalized with influenza (94, 96, 106). A multicenter, Phase III study performed in Korea, Japan, and Taiwan comparing single-dose i.v. peramivir (either 300 mg or 600 mg) to 5 days of oral oseltamivir in outpatients with uncomplicated influenza found similar times to alleviation of symptoms across all study groups (106). A Phase II study of 122 hospitalized patients with influenza randomized to receive one dose of i.v. peramivir (200 mg or 400 mg) or standard oseltamivir revealed no significant difference in time to clinical stability between groups (107). An interim analysis of a Phase III study comparing hospitalized patients with suspected influenza to either peramivir (600 mg i.v. once daily for 5 days) or placebo also showed no significant improvement in time to clinical resolution (42.5 hours vs. 49.5 hours, respectively, P = 0.97) (94). These results prompted early termination of the trial, and the agent has not been approved for this patient population. Despite these findings, there remain few options for hospitalized patients who are critically ill and cannot tolerate oral medications. In such circumstances, a longer course of daily peramivir may be appropriate (94). As there are not clinical trials directly comparing i.v. peramivir with i.v. zanamivir, the optimal agent and duration of therapy remains unclear.

RESPIRATORY SYNCYTIAL VIRUS

Palivizumab

Palivizumab (Synagis) is a humanized monoclonal antibody directed against an epitope in the A antigenic site on the F surface protein of respiratory syncytial virus (RSV). It is approved for the prevention of serious lower respiratory tract disease caused by RSV in high-risk pediatric patients. In a large, randomized, multicenter trial including over 1,500 high-risk patients, palivizumab prophylaxis resulted in a 55% reduction in hospitalization as a result of RSV (107). The American Academy of Pediatrics (2014) recommends RSV prophylaxis with monthly palivizumab (up to five doses) during RSV season for infants born before 29 weeks or patients who qualify on the basis of congenital heart disease or chronic lung disease of prematurity (109). In terms of safety, fever, rash, injection site reactions, and transaminitis are possible, but very few serious adverse events have been reported, and only 0.05% of patients experienced a hypersensitivity reaction deemed possibly or probably related to palivizumab (108, 110).

Ribavirin

Ribavirin (1-ß-d-ribofuranosyl-1,2,4-triazole-3-carboxamide, Virazole) (Fig. 5; Tables 1 and 2) is a synthetic nucleoside analog of guanosine approved in the United States in 1980 for treatment of RSV infections in hospitalized infants and young children, particularly those with severe lower respiratory tract RSV and underlying compromising conditions (e.g., prematurity, cardiopulmonary disease, or immunosuppression). In its oral form, it is approved for use in combination with interferon-α (IFN-α) for treatment of hepatitis C virus (HCV) (see Chapter 13 on Antihepatitis Virus Agents). It is available in oral formulations and solution for inhalation, as well as an investigational intravenous formulation.

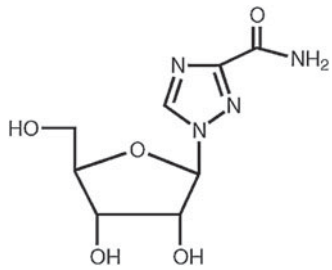

FIGURE 5 Molecular structure of ribavirin.

Inhaled ribavirin (Virazole) results in plasma concentrations ranging from 0.44 to 1.55 μM (with a mean concentration of 0.75 μM) when given via face mask for 2.5 hours daily for 3 days and has a half-life of 9.5 hours (Virazole prescribing information). Reported adverse effects include nausea, headache, worsening of bronchospasm, rash, and conjunctivitis, as well as precipitation on contact lenses (111).

Ribavirin is well known to be teratogenic and contraindicated in pregnancy; therefore, health care workers and pregnant women should be protected against exposure. It has been found to cause congenital anomalies of limbs, ribs, eyes, CNS, and death in offspring of rodents (112).

Controversy exists about the role of ribavirin for treatment of lower respiratory tract RSV infections in children (see Chapter 37 on RSV), because studies have not consistently shown virologic and/or clinical benefit in recipients and some have been criticized for design flaws (113). Ribavirin is also used to treat serious RSV infections in immunocompromised adults. Inhaled ribavirin has been found to decrease progression of upper respiratory tract disease to lower respiratory tract disease and mortality in adult hematopoietic cell transplant patients compared to no therapy (114). Either oral or inhaled forms of ribavirin may prevent progression of RSV infections from the upper respiratory tract to lower respiratory tract in bone marrow transplant patients (114, 115).

In addition to its systemic use in several viral hemorrhagic fevers (hantavirus hemorrhagic fever with renal syndrome, Lassa fever, Crimean-Congo hemorrhagic fever, Rift Valley fever), systemic ribavirin has been used in treatment of a number of respiratory viral infections, including influenza virus, parainfluenza virus, measles virus, and adenovirus, but its effectiveness remains unclear in these situations. Following encouraging initial reports, a controlled study of the oral triple-drug regimen of ribavirin, amantadine, and oseltamivir is progressing in influenza virus infection (116, 117). Ribavirin is active against human metapneumovirus *in vitro* and in animal studies, and one case report indicated a favorable outcome with i.v. ribavirin treatment in a lung transplant recipient with human metapneumovirus pneumonia complicated by respiratory failure and sepsis (118).

TABLE 3 Selected investigational (in the United States) antirespiratory virus agents in clinical development

Agent	*In vitro* spectrum	Mechanism of action	Stage of development in United States	Comments
Favipiravir (T-705)	Influenza A and B, many others including various filo-, flavi-, arena-, bunya-, alpha-, and enteroviruses	RNA polymerase inhibitor	Approved in 2014 in Japan only for outbreaks of novel or re-emerging influenza strains in which other influenza drugs are not effective or insufficiently effective. Phase III completed in 2016 in United States. Results pending	Oral administration. Phase II trial showed good tolerability and both antiviral and clinical efficacy in uncomplicated illness. Results of Phase III not yet published. Active against influenza strains resistant to current antiviral drugs and emerging strains such as A (H1N1) pdm09, A (H5N1), and A (H7N9) avian virus. Potentially synergistic with oseltamivir. Teratogenic in multiple species, contraindicated in pregnancy. Hyperuricemia. No data in severe influenza or children.
JNJ-872 (formerly VX-787)	Influenza A	Polymerase (PB2) inhibitor	Phase II	Oral administration associated with dose-related antiviral effects in experimental human influenza. Synergistic with NAIs in preclinical models. Phase II studies in uncomplicated illness and in combination with oseltamivir in hospitalized patients in progress.
Laninamivir (CS-8958)	Influenza A and B	Neuraminidase inhibitor (long acting)	Approved in Japan since 2010 for therapy and subsequently prophylaxis. In the United States, Phase II trial in adults with influenza A/B is completed	Long-acting NI (LANI) delivered by oral inhalation. Antiviral spectrum similar to zanamivir. In Japan, approved as single-day dosing for therapy. In the United States, the Phase II trial failed to achieve statistically significant primary endpoint of symptom alleviation in uncomplicated disease.
Nitazoxanide	Influenza A and B, many others including various flavi-, hepadna-, retro-, reo-, and caliciviruses.	Blocks the maturation of viral hemagglutin at the post-translational level	Phase III trial completed	Phase II study found reduced symptom duration and antiviral effects in uncomplicated illness. Phase III trial as monotherapy and in combination with oseltamivir completed; results pending.
DAS-181	Influenza A and B, parainfluenza and enterovirus EV-D68	Sialidase that removes host cell sialic acid receptors	Phase IIb for uncomplicated influenza Phase II for severe parainfluenza in immunocompromised patients	Delivered by inhalation. Modest antiviral and nonsignificant clinical effects in uncomplicated influenza. Case reports of antiviral activity and clinical benefit in severe PIV illness in transplant recipients; adequate tolerability.
Verdinexor (KPT-335)	Influenza A and B	Selective inhibitor of nuclear export. XPO1 antagonist	Phase I	Oral administration. Phase I completed, results pending.
AVI-7100	Influenza A	Interference with expression of the M1 and M2 genes	Phase I	Phosphorodiamidate morpholino oligomer containing three modified linkages (PMO*plus*). Active after intransal or intraperitoneal administration in ferret model of influenza. Intravenous administration in humans. Phase I estimated to be completed in November 2016.

(*Continued on next page*)

TABLE 3 Selected investigational (in the United States) antirespiratory virus agents in clinical development (*continued*)

Agent	*In vitro* spectrum	Mechanism of action	Stage of development in United States	Comments
Presatovir (GS-5806)	RSV	Fusion inhibitor	Phase II	Oral administration. In healthy adults experimentally infected with RSV, early treatment resulted in significant reductions of viral load, mucus weight, and symptom scores. Currently in Phase II in hospitalized elderly patients, lung transplant recipients and HSCT recipients with upper or lower respiratory infections due to RSV. Treatment emergent F gene mutations conferring reduced susceptibility to presatovir occur.
JNJ-678	RSV	Fusion Inhibitor	Phase IIa	Oral administration. As of April 2016, two studies have been completed—a multiple ascending-dose study in infants infected with RSV and an experimental RSV infection in healthy adult; results pending.
ALX-0171	RSV	Fusion inhibitor	Phase I/IIa	Inhaled antibody/nanobody that inhibits RSV replication by binding the F protein. Currently in Phase I/IIa trial of healthy infants and toddlers with RSV-related LRTI.
BTA-585	RSV	Fusion inhibitor	Phase II	Selective and potent RSV fusion inhibitor *in vitro* (EC50 = 8.4 nM for RSV A2).
ALS-8176 (pro-drug of AL-8112)	RSV	Polymerase inhibitor	Phase II	Orally administered nucleoside analog. In healthy adults experimentally infected with RSV, early treatment resulted in significant reductions of viral load, mucus weight, and symptom scores. Currently in Phase II in adults and Phase I in infants hospitalized with RSV infection. High threshold to development of resistance.
ALN-RSV01	RSV	Small interfering RNA to N gene	Phase II	Intranasal delivery demonstrated significant antiviral activity against experimental RSV infection in adults. Phase II trials of inhaled siRNA in RSV infected lung transplant recipients found trends toward reduced BOS at 180 days.
Brincidofovir (CMX-001)	Adenovirus, CMV, HSV, VZV, EBV, HHV-6A, HHV-8, BKV, JCV, HPV	DNA polymerase inhibitor	Phase III	Orally administered lipid ester prodrug of cidofovir with greater potency and low risk for nephrotoxicity. Dose-limiting gastrointestinal side effects. Preliminary data from Phase III trial on adenovirus infection in immunocompromised patients showed decreased viremia and mortality. Failed primary endpoint for CMV in Phase III.
rIFN-β (SNG001)	Rhinovirus, other respiratory viruses	Recombinant IFN-β1a	Phase II	Oral inhalation. A randomized, double-blind, placebo-controlled trial in asthmatics with a history of URTI-induced exacerbations reporting new URTI symptoms missed its primary endpoint (change in asthma symptoms assessed by an asthma control questionnaire) but reached this endpoint in subset of more severe asthmatics and resulted in better lung function and fewer severe exacerbations.
Vapendavir (BTA 798)	Rhinovirus, enterovirus (including polio)	Capsid inhibitor	Phase IIb	Orally administered twice daily. Initial Phase II study found trends toward fewer exacerbations in asthmatics with rhinovirus infections. Phase II trial of adult asthmatics with rhinovirus ongoing, estimated to be completed in August 2016.

NAI, neuraminidase inhibitor; PIV, parainfluenza virus; XPO1, exportin 1; RSV, respiratory syncytial virus; HSCT, hematopoietic stem cell transplantation; LRTI, lower respiratory tract infection; EC50, half-maximal effective concentration; BOS, bronchiolitis oblliterans syndrome; CNV, cytomegalovirus; URTI, upper respiratory tract infection.

Oral ribavirin along with corticosteroids was used extensively as an empirical regimen during the early stages of the global outbreak of severe acute respiratory syndrome (SARS) caused by SARS-associated coronavirus, but was later shown to lack *in vivo* antiviral activity or to provide clinical benefit (119, 120). Combinations of interferons and oral ribavirin have been used in Middle East respiratory syndrome coronavirus (MERS-CoV)-infected persons with no clear evidence for benefit (121–123).

INVESTIGATIONAL AGENTS

Research into new drugs against influenza is ongoing, but relatively few agents are in advanced stages of development (Table 3). These include the polymerase inhibitor favipiravir, which has activity against influenza viruses resistant to current classes of inhibitors as well as a number of other viruses (124–126), the polymerase PB2 inhibitor JNJ-872 (formerlyVX-787) (126, 127), the long-acting neuraminidase inhibitor laninamivir, already approved in Japan but with an uncertain future in the United States due to mixed results in a Phase II trial (128, 129), and nitazoxanide, which is being studied as both monotherapy or in combination with oseltamivir (130–132) (Table 3).

Development of anti-RSV agents seems to be near a breakthrough, with several new agents showing promising results in proof-of-concept studies during the last few years. The most advanced of these in clinical development include presatovir, a F protein inhibitor (133), and ALS-8176, a nucleoside analog that inhibits viral polymerase with a high threshold for resistance (134–136) (Table 3). Meanwhile, the inhaled sialidase DAS-181 is being tested for severe parainfluenza virus infections (137, 138) and brincidofovir (139) for the treatment of invasive adenovirus infections in immunocompromised hosts. The capsid binder vapendavir (140) and inhaled IFN-β (141) are also advancing in clinical trials for rhinovirus infections with focus currently on asthmatic patients. RNA interference (RNAi) targeting the N protein ALN-RSV01 has been evaluated in lung transplant recipients infected with RSV, with a particular focus on reducing development of chronic rejection in the form of bronchiolitis obliterans syndrome (142, 143).

Drug development for other respiratory viruses remains in the preclinical phase. GS-5734 is a RNA polymerase inhibitor shown to have not only therapeutic activity in rhesus macaques infected with Ebola virus but also *in vitro* activity against MERS and RSV (144).

The clinical development of monoclonal and polyclonal antibodies as therapeutic options against influenza and other respiratory viral infections shows much promise as well. A number of monoclonal antibodies targeting conserved epitopes on the HA stem have broad-spectrum neutralizing activities for influenza A viruses and are advancing in development. CR62621 is currently in Phase II in healthy volunteers infected experimentally with A (H1N1) pdm09 influenza virus. VIS410, which targets group 1 and 2 HAs, has shown therapeutic benefit for experimentally induced A (H1N1) pdm09 infection and is currently in Phase II trials (145–147). Among anti-HA stem monoclonal antibodies inhibitory for both group 1 and 2 HAs, MEDI8852 is in Phase Ib/IIa with or without oseltamivir and MHAA4549A (148) is currently in Phase II as monotherapy in uncomplicated influenza or in combination with oseltamivir for severe infections. One major concern with this approach is the potential for antibody-dependent enhancement of disease, but this has not been found in early clinical trials with MHAA4549A and VIS410 (126).

Among other viruses, the monoclonal anti-RSV antibody MEDI8897 has a lower EC_{90} than palivizumab (126) and was found to be safe in Phase II (149). REGN222, a monoclonal antibody against RSV-F, was well tolerated in Phase II trials (150) and is currently in a Phase III study assessing the safety and efficacy in preventing RSV among preterm infants during RSV season. Polyclonal antibodies against MERS-CoV, derived from bovines genetically modified to produce large quantities of fully human polyclonal IgG antibodies, have been shown to be safe and effective for therapy and prevention in mice (151), with Phase II studies currently being planned. Developing effective neutralizing monoclonal antibodies against SARS-CoV has been challenging, in part due to emergence of escape variants, even with administration of dual combination monoclonal antibodies (152). Apart from monoclonal antibody therapy, several studies have supported the use of convalescent plasma and hyperimmune globulin for influenza (153, 154), RSV (155), and SARS-CoV (156). The role for such an approach remains unclear, but several influenza studies are ongoing. Please refer to the pathogen-specific chapters for RSV (Chapter 37), Coronaviruses (Chapter 52), and Influenza (Chapter 43).

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Diagnosis of Viral Infections

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15

The clinical virology laboratory is an important and leading component of general microbiology that provides significant benefits to patient care. The traditional epidemiologic and academic reasons for diagnosis of viral infections have been expanded by rapid, often quantitative, assays that can impact on therapeutic management and public health decisions. This development is the result of many advances in diagnostic virology including improvement in cell culture (shell vial assays, mixed cell cultures, genetically engineered cell lines), availability of specific reagents such as monoclonal antibodies and, most importantly, the introduction of molecular techniques mostly based on polymerase chain reaction (PCR), which allow the sensitive and rapid detection of slowly growing or uncultivable viruses. The impact of the latter procedure is illustrated by the recent identification of several respiratory viruses including the human metapneumovirus (1), multiple coronaviruses including severe acute respiratory syndrome (SARS) (2) and Middle East respiratory syndrome (MERS) (3), human bocavirus (4), etc.

Apart from technological advances, the expanded role of the diagnostic virology laboratory can be also attributed to the increased pool of immunocompromised patients (e.g., HIV-infected individuals and transplant recipients) at risk of serious opportunistic viral infections and the increasing number of antiviral agents available for herpesviruses, HIV, influenza viruses, and hepatitis viruses. These factors have driven the development of rapid, sometimes point-of-care, diagnostic methods and new approaches including viral load measurement, antiviral drug susceptibility testing, and determination of viral genotypes and quasispecies.

Many laboratories now employ nucleic acid–based technologies as their main approach to virus diagnosis. Nevertheless, multiple methods for detecting virus infections remain in use in clinical laboratories. Viral culture, electron microscopy, histopathology, detection of viral antigens, and nucleic acids can be used to detect active viral infection, whereas serological testing is performed to assess the presence of virus-specific immune responses. Such diversity of methods has made it increasingly complex for physicians to request the appropriate procedure and to interpret the results. This necessitates excellent and frequent communication between the physician and the virology laboratory for the optimal use of diagnostic tests.

SPECIMENS

The performance of viral diagnostic tests is strongly influenced by the quality of the specimen that is received in the laboratory. Detection of viral pathogens is highly dependent on three important variables: 1) obtaining an adequate specimen from the appropriate site; 2) proper timing of specimen collection relative to onset of symptoms; and 3) effective and timely processing of the specimen. The specimen site should be determined by the clinical syndrome and the suspected viruses. The specific procedures for collecting clinical specimens are addressed in clinical laboratory manuals (5). The physician should be aware that the selection of specific clinical specimens could strongly affect the performance of a diagnostic test. This is particularly true for the diagnosis of respiratory virus infections, where nasopharyngeal aspirates or nasal washes are generally preferred to nasal or throat swabs. Also, the type of materials used for specimen collection could have an impact on test results. For example, dacron or rayon swabs are recommended in the case of vesicular lesions, since both herpes simplex virus (HSV) and varicella-zoster virus (VZV) have been shown to be inactivated by cotton and calcium alginate swabs (6, 7). In addition, flocked swabs can retrieve more epithelial cells than fiber (rayon) swabs for the detection of respiratory viruses (8). In general, nucleic acid–based diagnostic tests, because of their increased sensitivity, are more robust in terms of specimen quality.

For many acute virus infections, viral shedding begins shortly before the symptoms occur, peaks rapidly after the onset of symptoms, and then declines steadily as the illness resolves. Thus, in general, specimens should be collected as soon as possible after the onset of symptoms. However, there are some exceptions, such as for SARS-coronavirus infections in which viral load in respiratory specimens peaked at week 2 after the onset of symptoms (9).

Transport to the laboratory should be accomplished as soon as possible after specimen collection to ensure integrity. This is a critical requirement for cell culture procedures which require viable viruses but may be less relevant for detection of viral antigens or nucleic acids. In general, nonenveloped viruses (e.g., adenoviruses, enteroviruses) are more stable than viruses surrounded by a lipid envelope (e.g., VZV and respiratory syncytial virus [RSV]). Specimens other than blood should usually be kept at 4°C (wet ice) during

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transport, especially if transport will require more than one hour. If a delay of several days before testing is anticipated, the specimen should generally be frozen at a temperature of −70°C or lower (dry ice). However, freezing in a standard freezer (−20°C) is generally associated with greater loss of infectivity than holding at 4°C for several days and should be avoided. Swabs and tissue specimens should be placed in a viral transport medium (VTM) to preserve the quality of the sample. Many VTM formulations exist, which generally contain a salt solution to ensure appropriate ionic concentrations, a buffer to maintain pH, a source of protein for virus stability, and antibiotics/antifungals to prevent microbial contamination. Some nucleic acid tests (NATs) have their own special VTM, which have been optimized for specific specimens.

Laboratories should receive specimens after proper storage and transport and with relevant clinical information either on an appropriate requisition form or electronically. Key information that should accompany the specimen includes sampling site, time of specimen collection, the diagnostic concerns of the physician (possibly with suspected virus[es]) and accurate identifying information for the patient.

CELL CULTURE

All methods used for viral isolation require living cells because viruses are obligate intracellular parasites. Historically, the systems used to isolate viruses of medical importance consisted of laboratory animals, embryonated eggs, and cultured cells. However, most diagnostic virology laboratories no longer routinely perform viral isolation in either laboratory animals or embryonated eggs, but now rely solely on cell culture. Although it is gradually being replaced by immunologic and molecular methods that are either more rapid and/or more sensitive, cell culture retains the following advantages for viral diagnosis: 1) The method is relatively sensitive based on the inherent amplification of the virus during the replication process and is specific in that only the virus will be amplified; 2) viral culture has the potential to detect many different viruses if a sufficient number of sensitive cell lines are available to the laboratory. Therefore, one of the most important principles of designing a virus isolation protocol is the selection of cell lines that propagate important viruses from each sample type received by the laboratory; and 3) culture provides a viral isolate that can be further characterized if necessary (e.g., sero/genotyping, antiviral drug susceptibility testing).

There are also limitations to viral isolation for the diagnostic laboratory: 1) Certain viruses do not grow or grow very slowly in available cell lines so that the result is not clinically useful; 2) techniques for detecting a viral infection in lieu of virus isolation are more cost effective; and 3) successful isolation in cell culture depends on the viability of the virus in the specimen. However, several new approaches (enhanced cell culture) have shortened the time of virus isolation to more clinically useful timeframes and they will be discussed below.

Standard Cell Cultures

The utility of cell culture for use in viral isolation was pioneered by two important discoveries. First was the work of Gey who developed monolayer cell culture techniques (10). This was followed by the work of Enders, Weller, and colleagues who demonstrated that vaccinia virus (11) and polioviruses (12) could be grown and detected in roller tube cell cultures by characteristic cytopathic effects. Previously, the systems used for viral isolation had been either animal or embryonated egg inoculation. Following this landmark discovery, the number and type of cell lines that are useful for viral isolation have continued to expand and change.

The types of cell culture routinely used for viral isolation can be placed in one of three categories: primary cells, diploid (also called semi-continuous) cell lines, and heteroploid cell lines. Primary cells (e.g., monkey cells and chick embryo cells) are derived from tissue and have been propagated *in vitro* for the first time. These cells have the same chromosome number as the parental tissue and generally can only be subcultured once or twice. Diploid cell lines (e.g., human embryonic lung fibroblasts) develop during subculture of primary cells. A diploid cell line will consist of 75 to 100% of cells having the same karyotype as cells from the species of origin and can be subcultured 20 to 50 times prior to cell death. Heteroploid or continuous cell lines (e.g., HEp-2 and HeLa cells) exist as a population of cells with less than 75% of the population having a diploid chromosome constitution. The most important feature of this type of cell line is the ability of indefinite passages due to cell immortalization, which facilitates continuous access to cells for virus isolation. The two issues that have fostered the development of additional cultured cell lines for viral isolation are susceptibility (Table 1) and speed of replication (Table 2). Each cell culture type displays a differential susceptibility to each group or member of the virus families. Thus, multiple cell lines are required for a given sample type to detect the families of viruses capable of infecting a given organ system. In addition, isolation of viruses may not always use cell culture in monolayers. Suspension cultures of lymphocytes are used for isolation of retroviruses such as HIV-1 and 2, and several herpesviruses such as

TABLE 1 Susceptibility of cell culture types to commonly isolated human viruses[a]

Culture type	Viruses propagated
Primary	
Monkey kidney	Influenza, parainfluenza, enteroviruses
Rabbit kidney	Herpes simplex
Human embryonic kidney	Enteroviruses, adenoviruses
Diploid	
Fibroblast	Cytomegalovirus, varicella-zoster, herpes simplex, adenoviruses, rhinoviruses, enteroviruses (some)
Continuous	
HEp-2	Respiratory syncytial, adenoviruses, herpes simplex, enteroviruses (some)
A549	Adenoviruses, herpes simplex, enteroviruses (some)
LLC-MK2	Parainfluenza, human metapneumovirus
MDCK	Influenza
RD (rhabdomyosarcoma)	Echoviruses
Buffalo green monkey kidney	Coxsackieviruses

[a]Modified from reference (184) with permission from Elsevier.

TABLE 2 Time required to detect viruses in cell culture[a]

Virus	Number of isolates	Earliest day positive	Day when 50% positive	Day when 90% positive
Herpes simplex	512	1	1	3
Varicella-zoster	30[b]	3	7	13
Cytomegalovirus	116[c]	3	13	20
Adenovirus	125[d]	1	4	11
Enteroviruses	85	1	4	10
Respiratory syncytial	70[e]	2	4	7
Influenza	61[f]	2	4	7
Parainfluenza	104[g]	1	6	12
Rhinovirus	130	1	6	12

[a]Reproduced from (184) with permission from Elsevier. Data are from St. Louis Children's Hospital Virology Laboratory, 1997.
[b]12 of these were also detected on day 2 by shell vial assay.
[c]462 additional isolates were detected on day 1 or 2 by shell vial assay.
[d]18 of these were detected on day 1 by fluorescent antibody (FA) staining.
[e]734 additional specimens were positive on day 1 by FA staining or enzyme immunoassay.
[f]61 additional specimens were positive on day 1 by FA staining.
[g]30 additional specimens were positive on day 1 by FA staining.

Epstein-Barr virus (EBV), as well as human herpes viruses 6 and 7 (HHV-6 and HHV-7).

The cell culture systems used for viral isolation will, in general, depend on the type of specimen submitted to the laboratory. Traditionally, three cell types have been used for viral isolation: a primary cell culture such as a Rhesus monkey kidney, a diploid line such as a human embryonic lung, and a heteroploid line such as HEp-2. While this may still be a reasonable approach for some specimen types, it is no longer feasible or desirable for the isolation of all viruses. Due to cost and cell access considerations, some laboratories have replaced primary cells by a panel of continuous cell lines. It is also reasonable, both scientifically and financially, to use a single cell line when a specimen is submitted only for isolation of selected viruses such as HSV, cytomegalovirus (CMV), or VZV. The selection and types of cell cultures used in a laboratory offering virus isolation will depend on many factors, including the clinical or epidemiological need for viral isolation, the common specimen types received, cost to prepare or purchase the needed cell lines, and the availability of experienced personnel.

The general procedure for viral isolation starts with the processing of the clinical specimen. In most cases, this consists of the addition of antibiotics to the sample prior to inoculation of the appropriate cell lines. Each cell culture to be inoculated should be visually inspected prior to inoculation and only recently prepared cells should be used since older cell cultures are less susceptible to virus replication. The standard tube used for cell culture is a 16 × 125 mm screw-capped tube. These tubes facilitate incubation following sample adsorption in either a roller drum or in stationary racks. In either case, the system must slant the tubes at a 5 to 7° angle. In general, the inoculated tubes are incubated at 35 to 37°C, but lower temperatures (33°C) facilitate the isolation of some viruses (e.g., rhinoviruses). Some laboratories have replaced tubes by microwell plates for cell culture, which facilitates the use of multiple cell lines for optimal recovery of several viruses.

Cytopathic Effect

Each inoculated cell culture tube is examined with a light microscope to detect morphological changes typically daily for the first week and less frequently thereafter for up to 21 days. Morphological changes that are the result of viral replication are termed cytopathic effects (CPE). The observations of inoculated cell culture must be compared to sham-inoculated control monolayers from the same batch of cells. The CPE observed in cell culture may be characterized by the following: cell rounding, refractile cells, cell clumping, vacuolation, granulation, giant cells, syncytium formation, and cell destruction. The type of specimen, the cell line displaying CPE, and the type of CPE may be used as preliminary evidence for the identification of the replicating virus. For example, a respiratory sample inoculated into HEp-2 cells displaying large syncytia is consistent with the presence of RSV. A cerebrospinal fluid (CSF) inoculated into RD (rhabdomyosarcoma) cells displaying cell rounding and desintegration is consistent with an echovirus. A urine sample inoculated into fibroblast cells displaying isolated foci of swollen cells is consistent with CMV (Figure 1).

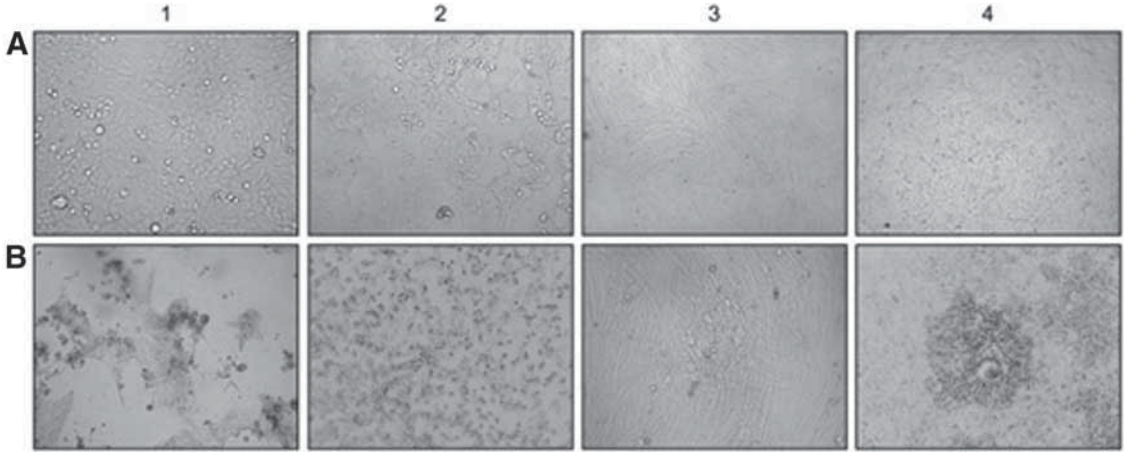

FIGURE 1 Cytopathic effects induced by some viruses. Panel A shows uninfected cell lines including HEp-2 (1), RD (2), fibroblast (3), and Vero (4) cells. Panel B shows the same cell lines infected with respiratory syncytial virus (1), enterovirus (2), cytomegalovirus (3), and herpes simplex virus (4).

The replication of certain viruses in cell culture may not produce any CPE. The replication of these agents can be detected by alternative techniques such as hemadsorption or interference assays. Hemadsorption is commonly used to detect the replication of orthomyxoviruses and some paramyxoviruses. Cells infected by influenza viruses, parainfluenza viruses, mumps and measles viruses display viral glycoproteins (hemagglutinin, hemagglutinin-neuraminidase) on their plasma membrane as an integral part of the process of viral replication. The viral glycoproteins promote attachment of certain species of red blood cells (e.g., guinea pig erythrocytes) to the infected cell membrane. Hemadsorption can be observed with a light microscope and, like CPE, is preliminary evidence that one of these viruses is replicating in the cell culture. Some other viruses, notably rubella, can be detected by taking advantage of the phenomenon of interference. For instance, when rubella grows in primary monkey kidney cells, the latter become resistant to challenge with an echovirus type 2 strain.

Identifying a virus replicating in a cell culture is confirmed using more definitive criteria of reaction with monospecific or monoclonal antibodies. Generally, these reagents are chemically conjugated to fluorochromes so that the reaction with a specific antibody can be determined by fluorescent microscopy. The immunofluorescence technique can be either indirect or direct. The indirect technique uses an unconjugated primary antibody to react with the infected cells followed by a fluorochrome-conjugated secondary antibody usually directed against the species specificity of the primary antibody. The direct technique consists of the primary antibody that is conjugated to a fluorochrome so that only a single staining and washing of the infected cells is necessary. Alternatively, some virology laboratories may use monospecific or pooled antisera to prevent the infection of susceptible cells, a process known as neutralization. Most virology laboratories use an immuofluorescence microscopy method for viral identification, whereas the neutralization method is primarily used to determine enterovirus serotypes.

In some clinical situations, the process of observing cells for CPE and then confirming that the CPE can be attributed to viral replication occurs too slowly. Over the past few decades, several enhanced cell culture methods have facilitated more rapid detection of viral replication.

Shell Vial Culture

The ability to detect viral infection prior to the development of CPE was first demonstrated with CMV (13). This method uses low-speed centrifugation to enhance the infection of human diploid embryonic fibroblast cells (MRC-5) in shell vials by CMV, followed by overnight incubation to establish the infection in which CMV-specific proteins assemble in the nucleus and, finally, detection of these by immunofluorescence microscopy after staining for an immediate early CMV protein. A shell vial is a vial containing a coverslip onto which a monolayer of cells has been grown. After centrifugation and incubation for 18 to 48 h, immunofluorescent staining is performed on the cells on the coverslip using monoclonal antibodies recognizing proteins that are expressed very early in the CMV replicative cycle. The speed and relative sensitivity of the shell vial system for CMV stimulated the application of shell vial systems for other viruses such as HSV, VZV, adenoviruses, and respiratory viruses. The detection of respiratory viruses requires inoculation of a greater number of shell vials with different cell types, increasing the incubation times, and using pools or cocktails of monoclonal antibodies capable of detecting each respiratory virus. Each antibody in the pool for detecting respiratory viruses has a unique staining pattern that can be used as a means to give a preliminary identification of the virus present in the shell vial monolayer. A second shell vial can then be subsequently stained with a single monoclonal to confirm the preliminary identification.

Mixed Cell Cultures

Viral isolation can be accomplished by using a shell vial cell culture with a mixture of cells in the monolayer. Such a mixture provides more susceptible cell types for a number of viruses, the low speed centrifugation enhances infectivity, and replication of the virus can be rapidly detected by immunofluorescence microscopy. The mixed cell systems have been developed to target viruses from specific types of specimen. The rapid respiratory virus mix, R-Mix (Quidel Corporation, San Diego, CA), is a combination of mink lung cells and A549 (human lung epithelial) cells (14) for detection of RSV, parainfluenza viruses, influenza viruses, and adenoviruses. An H&V-Mix is a combination of CV-1 (human kidney fibroblasts) and MCR-5 cells for detection of HSV, CMV, and VZV. The Super E-Mix is a combination of BGMK (buffalo African green monkey kidney) and A549 cells for detection of enteroviruses and other viral agents. The R-Mix with immunofluorescent staining performs comparably with either conventional tube culture or single cell shell vial culture with immunofluorescent staining but is generally more rapid (15). This system is a reasonable approach for laboratories that want to continue using shell vials for viral detection.

Genetically Engineered Cell Lines

Genetically engineered cell lines to be used for virus isolation were first developed for HSV (16). A baby hamster kidney cell line (BHK-21) was stably transformed with an HSV-inducible promoter (UL39 gene) attached to a functional *E. coli* beta-galactosidase gene. Infection of this cell line by either HSV 1 or 2 (specifically HSV proteins ICP0 and VP16) induces beta-galactosidase enzyme activity. Addition of a substrate, X-gal, for this enzyme results in the formation of a colored product in HSV-infected cells. This system is commercially available as an enzyme-linked virus-inducible system (ELVIS HSV ID; Quidel). The advantages of this system include rapid detection with a result available after overnight incubation followed by addition of the substrate; visual signal by detection of either CPE and/or the blue color; and adaptability to large volume laboratories and potential automation. A modification of the ELVIS system now allows subsequent staining directly on the monolayer with type-specific, fluorescent-labeled HSV monoclonal antibodies. The concept of virus specific indicator cell lines may be feasible for other viruses (17), and a format has been developed for rapid HSV antiviral susceptibility testing (18).

The measles virus can be difficult to grow in cell cultures. This is believed to be due to strains of measles having tropism for different host cell receptors. A Vero cell line, stably transformed with the receptor hSLAM (human Signaling Lymphocytic Activation Molecule), has proven ideal for growing measles virus from patient specimens (19).

ANTIVIRAL SUSCEPTIBILITY TESTING

Introduction

In the past two decades, safe and effective antiviral drugs have been developed for the treatment of many acute and chronic viral infections. Not surprisingly, the increased and

TABLE 3 50% inhibitory concentration (IC_{50}) cutoffs for herpes simplex virus and cytomegalovirus antiviral resistance using the plaque reduction assay

Virus	Drug	Susceptible	Intermediate	Resistant
Herpes simplex virus	Acyclovir	<9 μM		≥9 μM
	Foscarnet	<330 μM		≥330 μM
Cytomegalovirus	Ganciclovir	≤6 μM	6–12 μM	>12 μM
	Foscarnet	≤400 μM		>400 μM

Note: Resistance is also defined by a greater than 2- to 3-fold increase in IC_{50} values compared to a pretherapy isolate or a wild-type control virus.

prolonged use of these drugs, most notably in immunocompromised patients, has been accompanied by the emergence of drug-resistant viruses. However, clinical failure may not only be due to the presence of a drug-resistant virus but can also depend on factors such as the patient's immunological status, compliance, and the pharmacokinetics of the drug in that patient. Examples of clinical situations which warrant the use of antiviral susceptibility testing for herpesviruses include failure of HSV or VZV cutaneous lesions to resolve, the appearance of new lesions while on acyclovir (valacyclovir or famciclovir) therapy (20, 21), and progressive retinal or visceral CMV disease during ganciclovir (valganciclovir) therapy (22, 23). Ganciclovir-resistant CMV infections were first reported among HIV-infected subjects with CD4 T cells of $<50 \times 10^9$/l (24) and are now an important problem in solid organ transplant recipients, particularly in the context of a primary infection after organ transplantation (CMV-seropositive donor and CMV-seronegative recipient) (25, 26). Also, continuous influenza shedding during therapy with amantadine/rimantadine or the neuraminidase (NA) inhibitors (oseltamivir/zanamivir) in immunocompromised patients should lead to suspicion of antiviral drug resistance and may warrant antiviral testing (27, 28). Resistance testing for clinical management of HIV-1 infection is recommended at entry into care, in the case of treatment failure (worsening clinical disease, increase in viral load, decrease in CD4 T lymphocytes), primary infection, and pregnancy (29–31). Several recent review articles have been published on the mechanisms of antiviral drug resistance for herpesviruses (32, 33), influenza (34), and HIV-1 (35).

Phenotypic Assays

Phenotypic assays measure the effect of an antiviral drug on the growth of a virus. This can be determined by infectivity (e.g., plaque or yield reduction) assays, viral antigen or viral nucleic acid production, and enzyme activity. Such assays directly measure and quantify the effect of antiviral drugs on viral growth. However, they generally require isolation and passage of the virus in cell culture, followed by viral titration before antiviral drug susceptibility testing begins. Thus, these tests tend to be slow, labor intensive, and difficult to standardize. In addition, *in vitro* passages can introduce unintended viral mutations. Phenotypic results are typically expressed as the drug concentration that inhibits 50% or 90% of viral growth relative to a no-drug control (IC_{50} or IC_{90}). Importantly, because these assays have been difficult to standardize, resistant and susceptible control strains should be tested with each batch of clinical isolates. Phenotypic antiviral susceptibility assays are currently in use for herpesviruses (HSV, VZV, CMV), influenza viruses, and HIV-1. A standard procedure for HSV antiviral susceptibility testing by plaque reduction assay (PRA) has been approved by the Clinical and Laboratory Standards Institute (formerly NCCLS) in 2004 (36). A similar PRA has been described for CMV (37).

The PRA has classically been the standard method of susceptibility testing to which new methods are compared. In this assay, a standardized viral inoculum is added into multiple wells of a plate containing susceptible cells and serially decreasing concentrations of an antiviral agent. A solidifying agent such as agarose is usually added to the cell culture medium to allow the formation of discrete viral plaques. Following an incubation period (which varies according to the virus), the plates are fixed and stained with a dye (crystal violet) and the plaques are counted. The IC_{50} and sometimes the IC_{90} values are determined and compared to those obtained for susceptible and resistant control strains. Breakpoint resistance values have been proposed for HSV and CMV (Table 3) although it should be emphasized that many variables can affect the results such as the cell line, the viral inoculum, the incubation period, etc. For a slow-growing virus such as CMV, the time involved for initial growth, viral stock preparation, and antiviral susceptibility testing is >6 to 8 weeks, whereas it can take <2 weeks in the case of HSV. Another drawback of the PRA is the possibility of selecting some genotypes in a mixed viral population ("quasispecies") during the initial passages in cell culture in the absence of drug (38).

Other phenotypic assays (see more detailed procedures in (39)) include the dye uptake assay for susceptibility testing of HSV (40). This is a colorimetric method that involves the quantification of a vital dye (neutral red) by viable cells but not by infected (nonviable) cells. The assay is semiautomated and is reproducible with the use of 96-well microtiter plates and a spectrophotometer. A DNA hybridization assay (Hybriwix assay, Diagnostic Hybrids, Inc., Athens, OH) was developed to measure HSV, CMV, and VZV drug susceptibilities (41) but this assay is no longer commercially available. Reporter genes (e.g., luciferase or alkaline phosphatase) can also be introduced in cell lines (42) or recombinant viruses (43) allowing high throughput testing.

For influenza phenotypic susceptibility testing to the NA inhibitors (zanamivir, oseltamivir), the currently preferred method consists of directly measuring inhibition of NA activity (44). After incubation of the virus containing the NA with different concentrations of NA inhibitors, a fluorescent or chemiluminescent substrate is added, then fluorescence or chemiluminescence is quantitated. The working group on surveillance of influenza antiviral susceptibility has proposed cutoff values for influenza testing against NA inhibitors (Table 4) (45). Of note, the NA assay cannot detect drug resistance caused by the presence of hemagglutinin mutations, which have been less commonly reported than NA mutations (46).

Phenotypic assays for susceptibility testing of HIV-1 were traditionally performed in cell culture using primary

TABLE 4 Breakpoints for influenza virus susceptibility testing against the neuraminidase inhibitors[a]

Virus	Reduced inhibition[b]	Highly reduced inhibition[b]
Influenza A	10–100×	>100×
Influenza B	5–50×	>50×

[a]Based on the recommendations from the working group on surveillance of influenza antiviral susceptibility (45).
[b]Fold increase in IC_{50} values compared to wild-type (control) virus.

peripheral blood monoclear cells from HIV-1-negative donors (47). In this assay, virus replication in the presence or absence of an antiretroviral drug is monitored by quantifying the HIV-1 p24 antigen using enzyme immunoassay (EIA)-based tests. Nowadays, recombinant virus phenotypic assays (48) involve insertion of the reverse transcriptase, polymerase and integrase genes of HIV-1 from a patient into a vector consisting of a rapidly replicating laboratory viral strain that also contains a reporter gene (e.g., luciferase) to measure viral growth in the presence of a drug compared to a wild-type virus. Some recombinant virus assays have been developed commercially such as the PhenoSense assay (Monogram BioSciences-LabCorp) and the Antivirogram assay (Tibotec-Virco, now discontinued) (49). Other types of phenotypic assays beyond the scope of this chapter include the yield reduction assay (50), EIA-based tests (51), and flow cytometry testing (52).

Genotypic Assays

Genotypic assays allow for the rapid detection of genetic mutations associated with antiviral drug resistance. Most of these tests first involve a round of nucleic acid amplification for the specific viral genes implicated in resistance to a certain drug, followed by direct sequencing of the amplified product, and finally comparison of the viral sequence to that of a pretherapy isolate or a reference strain. These assays are particularly useful for detecting evidence of resistance, when a discrete number of characterized resistance mutations are known. Genotypic assays have been most widely used for detecting ganciclovir-resistant CMV or antiretroviral-refractory HIV-1 infections, since phenotypic assays for these viruses are slow and tedious. However, such tests have also been used to distinguish genetic variants of hepatitis B virus (HBV) associated with lamivudine resistance (53), M2 mutants associated with influenza A resistance to amantadine (54), NA and/or hemagglutinin influenza mutations associated with resistance to NA inhibitors (55, 56), and HSV or VZV mutations conferring resistance to acyclovir (20, 21). In the latter case, development of genotypic assays has been hampered by the fact that the thymidine kinase and DNA polymerase genes of herpesviruses are highly polymorphic and that drug-resistance mutations are scattered throughout the gene. Also, phenotypic assays for HSV are generally more convenient due to rapid growth of this virus (typically in 24 to 48 h).

Genotypic assays for CMV have been developed mainly to detect UL97 (protein kinase) and also UL54 (DNA polymerase) mutations associated with ganciclovir (or valganciclovir) resistance (Figure 2). PCR amplification of a short fragment of the UL97 gene followed by either restriction endonuclease digestion or direct sequencing have been used to detect the most frequent ganciclovir resistance mutations at codons 460, 520, 594, and 595, which are responsible for approximately 80% of resistance cases (57, 58). The major advantage of this method consists of its rapidity since resistance-associated mutations can be detected directly in clinical samples such as whole blood, leukocytes, plasma, urine, etc. within a few days after sample collection (23). For more comprehensive analysis of ganciclovir resistance but also to detect CMV resistance to cidofovir and foscarnet, PCR amplification and DNA sequencing of the catalytic regions of the UL54 gene must also be undertaken. These genotypic assays can be performed by commercial laboratories such as Viracor-IBT. However, the genetic map of CMV UL54 mutations associated with drug resistance has not been completed yet and an important problem consists of discriminating between polymorphic (natural variations) and resistance mutations. Recombinant viruses based on bacterial artificial chromosomes (BAC) or overlapping cosmids must be generated to definitively determine that a particular mutation is associated with drug resistance (59, 60), which is beyond the scope of the general virology laboratory.

The development of HIV resistance to all antiretroviral agents is a significant clinical problem. Direct sequencing of PCR-amplified reverse transcriptase, protease, or integrase genes is considered to be the gold standard for assessing HIV-1 drug resistance (reviewed in (29, 35, 61)). Commercially available FDA-approved sequencing-based methods for HIV genotyping include for instance the Trugene HIV-1 (Siemens Healthcare GmbH, Erlangen, Germany) and the ViroSeq HIV-1 (Abbott Molecular, Des Plaines, IL) assays. Both systems are similar with respect to the complexity of the assays and have a detection limit of ≈20% for minor quasispecies according to the manufacturers' instructions. Hybridization techniques, as an alternative to complete sequencing, may be used to detect specific mutations associated with drug resistance. In the INNO-LiPA HIV-1 line probe assay (Fujirebio Europe N.V., Zwijnaarde, Belgium), codon-specific oligonucleotide probes are applied as discrete lines on a nitrocellulose membrane in a strip format. Hybridization of biotin-labeled test isolate amplicon with

FIGURE 2 Cytomegalovirus (CMV) UL97 mutations associated with ganciclovir resistance. CMV UL97 conserved regions are represented by shaded boxes. Numbers under the boxes indicate the positions (codons no.) of these conserved regions. Vertical bars indicate the presence of amino acids substitutions while the hatched box indicates a region (codons 590 – 607) in which diverse deletions (from 1 to 17 codons) have been reported.

the probes for specific codons (wild-type and mutants) leads to the production of color in the presence of avidin enzyme complex and substrate. Some researchers have concluded that the clinical utility of LiPA is limited by the high rate of indeterminate results (62) and this test is no longer commercially available. In another hybridization method, the microarray chip-based technology, over 16,000 unique oligonucleotide probes are used for hybridization with fluorescein-labeled target nucleic acid (HIV PRT GenChip, Affymetrix, Inc., Santa Clara, CA). This method was shown to be less reliable as compared to dideoxynucleotide sequencing and is no longer commercially available (63). The final step in the process of genotypic resistance testing is the analysis of generated sequences. Interpretation of the genotype can be based either on rule-based algorithms or on "virtual phenotypes." The sequences may be aligned using different software programs included in commercially available genotypic assays or with on-line databases. In "virtual" phenotyping, the genotypic mutation profile of a viral strain is compared with the available paired genotypes and phenotypes in the database.

In summary, phenotypic or genotypic assays can be used depending on the virus and the clinical situation. Phenotypic assays offer the advantage of providing a direct measurement of viral susceptibility to any antiviral drugs and of quantifying the level of resistance. On the other hand, genotypic assays offer the distinct advantages of greater speed and efficiency in analyzing a large number of viral strains, but only identify known drug resistance mutations. Discordance between phenotypic and genotypic assays can result from the presence of viral quasispecies (genotypic mixture of wild-type and mutant viruses) and the presence of complex patterns of mutations (resistance and compensatory mutations). In recent years, the advent of next-generation sequencing has resulted in a lowering of the threshold for detecting minor resistant subpopulations from 20% to 1 to 2% compared to conventional (Sanger) DNA sequencing. This promising technology (see section on nucleic acid tests) has already been used in the context of detecting CMV (64), influenza (65), and HIV-1 (66) drug resistance mutations in clinical samples.

DIRECT DETECTION OF VIRUS OR VIRAL ANTIGEN

With the increasing availability, decreasing cost, and improved turnaround time of molecular techniques for the detection of viral nucleic acids, the use of direct detection methods for virus or viral antigen has diminished in the clinical laboratory. Despite their generally lower sensitivity than molecular tests, some clinical laboratories may continue to offer these tests when expertise in molecular techniques is lacking, cost of molecular tests is prohibitive, and/or test requests for certain viruses are too low in number to justify in-house use of molecular tests.

Immunoassays

Of all the nonmolecular techniques for the direct detection of viruses or viral antigens within clinical specimens, immunoassays remain the most widely used. These assays utilize antibodies (antisera) which may be monoclonal or polyclonal directed against a specific viral antigen or antigens (67). The resulting antibody–antigen complexes can be detected using a number of different techniques ranging from direct visualization methods to those that detect viral antigens using solid-phase immunoassays (SPIA) such as EIA and enzyme-linked immunosorbent assays (ELISA).

Direct Visualization Immunoassays

Visualization of viral antigens within tissue samples or individual cells is most commonly achieved by either direct or indirect staining methods (68). Not only can one detect the presence or absence of viral antigens within cells, one can also determine the location (e.g., nucleus, cytoplasm, inclusion bodies, etc.) of the viral antigens. In the direct method, the antibody (antiserum) directed against the virus of interest is conjugated with either an enzyme (e.g., horseradish peroxidase, alkaline phosphatase, others) or a fluorescent label (e.g., fluorescein isothiocyanate [FITC]). The antibody is added onto a glass slide to which the patient sample has been fixed. Once the antibody–antigen reaction is allowed to occur, a substrate is added for the conjugated enzyme, which, when acted upon by the enzyme, results in a color reaction that is visible using a light microscope. If the antibody label used is FITC, the presence of antibody–antigen complexes can be visualized using an immunofluorescence microscope set at the appropriate wavelength. In the indirect method, the initial antibody (e.g., mouse monocolonal antibody) directed against the virus of interest is not conjugated to an enzyme or a fluorescent label. After it is allowed to bind to the virus or viral antigen (if present in the patient sample), a second conjugated or labeled antibody directed against the first (e.g., goat anti-mouse antibody) is allowed to bind to the antibody within the antigen–antibody complex. Visualization is then performed as for the direct method. The advantage of the indirect method is that it can "amplify" the signal because many secondary antibodies may bind to the initial antibody resulting in a stronger signal.

Fluorescent antibody immunoassays including direct immunofluorescence (DFA) and indirect immunofluorescence (IFA) microscopy continue to be used in diagnostic virology because they are relatively easy to perform and inexpensive (Table 5). They are applicable to a wide variety of specimen types and can be used for the detection of many different viruses or viral antigens with high specificity, particularly when monoclonal antibodies are used. Pooling of monoclonal antibodies, each labeled with a different fluorescence molecule, allows for the simultaneous detection of different viruses in the same sample. For example, a pool of monoclonal antibodies directed against influenza viruses A and B, RSV, parainfluenza viruses 1, 2, and 3, and adenovirus is often used to screen respiratory specimens such as nasopharyngeal swabs and washings. If the screen is positive, then individual slides can be prepared and stained using separate antibodies to identify the specific virus or viral antigen present in the specimen. Fluorescent antibody staining is used in many laboratories as initial screening for HSV and VZV in skin and mucous membrane samples, and for respiratory viruses in a variety of respiratory specimens (69, 70). Detection of CMV pp65 antigens using direct immunofluorescent staining of peripheral blood leukocytes (PBLs) continues to be used in many centers as an alternative to molecular techniques for the detection and quantitation of CMV in blood specimens. By using a fixed number of PBLs per slide, one can provide a measure of the number of positive fluorescent cells per fixed number of PBLs which can be used to monitor CMV activity in patients with primary or reactivated CMV infection/disease following organ and bone marrow transplantation (71). More recent guidelines, however, recommend that molecular assays be used in place of immunostaining techniques (72).

TABLE 5 Detection of viral antigens by direct fluorescent antibody (DFA) staining

Clinical presentation	Possible viral agents detectable by DFA	Specimen source
Colorado tick fever	Colorado tick fever virus	Blood (erythrocytes)
Congenital infections	Rubella, CMV, HSV	Nasopharyngeal, throat, lesion scrapings, tissue
Conjunctivitis, keratitis	HSV, adenovirus, measles virus	Conjunctival cells, corneal scrapings
Disseminated disease, immunocompromised host	HSV, CMV, VZV, EBV	Tissues, lesion scrapings
Encephalitis	HSV	Brain biopsy
	Rabies virus	Brain tissue
	Mumps virus	Throat, urine sediment
Macular or maculopapular exanthems	Measles virus, rubella virus, mumps virus, adenovirus	Nasopharyngeal, throat, urine sediment
Mucocutaneous vesicles or ulcers	HSV, VZV, adenovirus	Scrapings from base of the lesion, mucosal cells
Respiratory infection	Influenza A and B viruses, parainfluenza viruses, adenovirus, RSV, human metapneumovirus, measles virus, mumps virus	Nasopharyngeal swabs, washes, or aspirates; throat swab; lung biopsy; bronchoalveolar lavage, wash, or brushing

Solid Phase Immunoassays

Solid phase immunoassays for the direct detection of viral antigens in clinical specimens include EIA, ELISA, latex agglutination (LA), membrane-immunoassays (also known as lateral flow immunochromatographic assays), and radioimmunoassay (RIA) (67). In many laboratories, EIAs and ELISAs remain the predominant solid-phase immunoassay techniques used for viral diagnosis. Many EIAs and ELISAs have been automated which has improved consistency in the performance of these tests, objectivity in the interpretation of the results, as well as increased throughput and reduced turnaround times. Although the terms EIA and ELISA have slightly different meanings, they are often used interchangeably to denote any assay that utilizes an enzyme rather than radioactivity as the reporter label. For simplicity, ELISA will be used in the remainder of this section.

ELISAs can be used to detect either antigens or antibodies in a clinical sample (73). The most common ELISA formats for the detection of viral antigens include direct, sandwich, and competitive ELISAs. It should be noted that there are slight differences in the literature with respect to the use of these terms with some overlap between the term '*indirect*' ELISA and '*sandwich*' ELISA. In the direct ELISA method for the detection of viral antigens, the surface of a solid phase (e.g., the wells of a polystyrene microtiter plate) is coated with primary antibodies directed against the antigen of interest. When the sample is added to the solid phase, the specific viral antigen of interest, if present, will bind to the primary antibody forming an antigen–antibody complex that is fixed to the solid phase. Once the excess sample is removed by several washing steps, a second antibody conjugated with a reporter enzyme is added and allowed to also bind to the antigen of interest. Detection is achieved by the addition of a substrate (appropriate for the reporter enzyme) which when acted upon, results in a color reaction. Different reporter enzymes can be used including horseradish peroxidase, alkaline phosphatase, beta-glucosidase, and others. The presence and intensity of the color generated is then read using a spectrophotometer which yields the result as an optical density (OD) value. With standardization of the assay, the OD value can be converted into a quantitative value and reported in international units per milliliter (IU/ml).

A sandwich ELISA for the detection of viral antigen(s) is performed in essentially the same way as a direct ELISA except that the secondary antibody is not conjugated with an enzyme. Instead, once the secondary antibody is allowed to bind to the antigen, an enzyme-conjugated antibody directed against the Fc region of the secondary antibody is added. This is then followed by the addition of an appropriate substrate. By using an enzyme-linked antibody that binds to the Fc region of other antibodies, this same enzyme-linked antibody can be used in a variety of different assays for different antigens, thus making them somewhat universal.

For competitive ELISAs, the primary antibody directed against the antigen(s) of interest is mixed with the patient sample directly resulting in the formation of antibody–antigen complexes. These antibody–antigen complexes are then added to an antigen-coated well in a polystyrene mitcrotitre plate. Following incubation, the plate is washed in order to remove unbound primary antibodies. In general, the more antigens present in the patient sample, the more antigen–antibody complexes are initially formed, leaving fewer unbound antibodies available to bind to the antigens fixed to the wells of the polystyrene microtitre plate. The plate is again washed after an incubation period to remove any remaining unbound primary antibody. This is followed by the addition of a secondary enzyme-conjugated antibody, which is directed against the primary antibody followed by the addition of an appropriate substrate. In a competitive ELISA therefore, if specific antigen was present in the original patient sample, the signal generated is reduced and thus the OD value is low.

The solid phase can be a membrane rather than a microtiter plate. This format has been developed for detection of such viral antigens as influenza virus, RSV, rotavirus, and others (74). Modifications of the standard EIA and ELISA methods by the use of tiny beads (microparticles) to increase the surface area for the coating of antigens or antibodies, thus potentially increasing sensitivity, has led to the introduction of micro-enzyme immunoassays (MEIA). This has resulted in a shortening of the time required to complete the

assay from 3 to 4 hours for standard ELISAs to less than one hour.

Chemiluminescent immunoassays (CLIA) and chemiluminescent micro-enzyme immunoassays (CMIA) follow the same general principle as ELISAs, but use an enzyme/substrate combination that results in the generation of emitted light rather than a color signal (75). The emitted light is detected using a luminometer which generates a value reported as relative light units (RLU). Regardless of whether a color reaction is produced or a light signal is generated, the signal strength is typically proportional to the amount of antigen present in a sample. However, not all assays are quantitative and therefore one cannot assume that a high OD or RLU value always correlates with the presence of more antigens in the patient sample. Some assays include quantitation standards which are used to generate a standard curve for quantifying the amount of antigen in a patient sample.

ELISAs are very sensitive and are capable of detecting antigens at the picomolar to nanomolar range (10^{-12} to 10^{-9} moles/l) (73). However, chemiluminescent assays tend to be even more sensitive and have a wider dynamic range than standard ELISAs. ELISAs have been developed for the detection of numerous different viral antigens. Most assays have been developed and validated using a limited number of different sample types (e.g., serum and/or plasma) and their use for detection of antigens in other sample types such as CSF or other body fluids may give erroneous results. Non-specific or cross-reactions can occur with ELISAs depending on whether the antibodies used in the assay are monoclonal or polyclonal and how similar (or different) the antigens are amongst different viruses (e.g., HSV-1 and HSV-2) (70).

Histopathology/Cytopathology

Standard light microscopy examination of stained clinical material may identify direct cellular changes which may be the first evidence of a viral infection. For some viruses, infected cells and tissues may exhibit cytological alterations that are pathognomonic for a specific virus (Table 6), while for others, the changes may be nonspecific and simply raise the possibility of a viral infection. Some stains allow for the detection of viral inclusions, but cannot provide a definitive identification of the specific viral agent. During viral latency and possibly at other times during viral replication, cells may appear entirely normal with no histologic changes to suggest a viral infection. Therefore, one cannot rule out a viral infection using histopathology/cytolopathology methods alone.

None of the usual cytologic stains (e.g., hematoxylin and eosin [H & E], Wright-Giemsa, Giemsa [Tzanck preparation], Papanicolaou) is specific for detecting the presence of a viral infection and all lack sensitivity (76). For example, the classic Tzanck preparation cannot be used to distinguish HSV from VZV infections, an important clinical distinction required for proper management, infection control, and counseling of patients.

One of the key areas where cytological examination plays an important role has been the Pap smear for cervical scrapings as part of screening for early changes indicative of cervical cancer (77). Virtually all cervical cancers are associated with specific oncogenic subtypes of human papillomavirus (HPV). Thus the presence of atypical squamous cells of undetermined significance (ASCUS) and atypical glandular cells of undetermined significance (AGUS) coupled with newer molecular tests which can detect the presence of most of the oncogenic subtypes of HPV is rapidly becoming the standard for managing women for cervical cancer prevention.

In renal transplant patients, up to 10% of recipients may develop polyomavirus-associated nephropathy (PVAN) which may result in loss of the renal graft. Histological diagnosis using light microscopy plays an important role in these patients (78). However, because the pathological changes of PVAN are heterogeneous and the intranuclear viral inclusions are indistinguishable from other viruses such as adenovirus and herpesviruses, immunohistochemical staining, electron microscopy, or molecular testing is required for making a definitive diagnosis. Similarly, although cytological examination of cells shed in urine may identify "decoy cells" which are cells containing intranuclear inclusions, it cannot distinguish between the different types of polyomavirus (e.g., BK versus JC) or from adenovirus-infected cells (79).

Cytological examination may also play a role in distinguishing viral shedding from active infection in some clinical situations. For example, the presence of cytological changes, including the presence of cytomegalic inclusion bodies in a bronchoalveolar lavage (BAL) sample, is more suggestive of active CMV infection than the detection of CMV in the same sample without evidence of cytological changes. The latter may simply represent asymptomatic shedding.

Electron Microscopy

In most diagnostic laboratories, electron microscopy (EM) for the diagnosis of viral infections has been supplanted by other methods, and many reviews of specific diagnostic recommendations fail to even mention the potential role of EM. Some laboratories may continue to use EM for screening stool samples for viruses associated with acute gastroenteritis because of the wide array of potential viral pathogens that may be detected, and the lack of a simple, single assay for detection of the multiple viral pathogens (80, 81). EM also plays an important role in the detection of BK-polyomavirus in tissue biopsies and urine samples from renal transplant patients with suspected BK-virus associated nephropathy (78, 82).

Virtually any specimen type can be examined by EM for viruses. In general, liquid samples such as CSF, saliva, tears, urine, vesicle fluid, etc. may be used directly, while others may need to be rehydrated (e.g., dried tissue samples) or clarified (e.g., fecal material) before examination by EM. Specimens to be examined by EM must first be adsorbed onto a thin plastic and/or carbon film adherent on the surface of an EM grid before staining (83, 84). In order to improve the sensitivity of EM, the use of either pooled human immunoglobulins or specific antibodies during the adsorption step can be used (e.g., solid-phase immunoelectron microscopy [SPIEM]). In addition, ultracentrifugation of the original sample followed by re-suspension in a smaller volume or direct centrifugation of the sample onto the EM grid may also help increase the probability of detecting virus particles by EM. Once the grid has been prepared with the specimen, it is subjected to either positive or negative staining. Both methods use heavy metal ions (e.g., lead, tungsten, and uranium ions) to generate sufficient image contrast and resolution (84). Because positive staining is technically more demanding and requires considerably more time to perform, it is impractical for routine clinical specimens and has been replaced by negative staining. The most common negative stains are 1% aqueous uranyl acetate and 1% phosphotungstic acid. These electron-dense stains penetrate the virion and provide contrast for visualization of the

TABLE 6 Cytological changes associated with selected viral infections

Virus	Clinical presentation	Cytological findings
Adenovirus	Upper respiratory tract infections, pneumonia, acute keratoconjunctivitis	Small multiple eosinophilic intranuclear inclusions (early); large single dense basophilic intranuclear inclusions ("smudge cells," late)
	Hemorrhagic cystitis	Dense basophilic intranuclear inclusions in transition cells
BK virus	Urethral stenosis (renal transplant patients) and interstitial nephritis (immunosuppressed patients)	Large full mucoid intranuclear inclusions (early); dense full basophilic inclusions bulging from cytoplasm (late)
Cytomegalovirus	Pneumonia	Cytomegaly; large, single amphophilic intranuclear inclusions (Cowdry A); small basophilic intracytoplasmic inclusions
Herpes simplex virus	Herpes genitalis, tracheobronchitis, corneal vesicle, or ulcer	Large ground-glass nucleus (early); eosinophilic intranuclear inclusions (late) with peripheral chromatin condensation; multinuclearity with nuclear molding
	Generalized infection or local cystitis	Ground-glass nuclei (early); eosinophilic intranuclear inclusions (late); multinuclearity; may be part of tubular cast
Human papillomavirus	Condyloma acuminatum, cervical dysplasia	Enlarged hyperchromatic nucleus; rare basophilic intranuclear inclusions; perivascular cytoplasmic clearing and vacuolar degeneration (koilocytic change)
Human polyomavirus	Progressive multifocal leukoencephalopathy (PML)	Enlarged oligodendrocytes with enlarged nuclei containing large basophilic inclusions
	Polyomavirus-associated nephropathy (PVAN)	Enlarged basophilic inclusions and displaced chromatin in renal tubular epithelium and urothelium; basophilic ground-glass nuclear inclusions in epithelial cells ("decoy cells") in urine
Measles virus	Prodromal	Mulberry-like clusters of lymphocytic nuclei in nasal secretions
	Measles with exanthema	Multinucleated giant cells with intracytoplasmic and intranuclear inclusions
Molluscum contagiosum	Reddish papular lesions of eyelid or conjunctiva	Large dense basophilic intracytoplasmic inclusions displacing the nucleus
	Vaginal, penile, or perineal papule with central umbilication	Large dense basophilic intracytoplasmic inclusions displacing the nucleus; squamous cells often bean-shaped
Nipah virus	Encephalitis	Intracytoplasmic eosinophilic inclusions
Parainfluenza virus	Bronchitis	Cytomegaly; single or multiple nuclei; small eosinophilic intracytoplasmic inclusions
Parvovirus B19	Aplastic crisis, hydrops fetalis	Nuclear inclusions in erythroid precursor cells, bone marrow, or liver (fetus)
Respiratory syncytial virus	Tracheobronchitis, pneumonia	Large multinucleated cells; intracytoplasmic basophilic inclusions with prominent halos
Varicella-zoster virus	Vesicular eruptions in dermatome (shingles) or accompanying varicella	Multinucleated cells with intranuclear eosinophilic inclusions

surface detail of virus particles. Negative staining allows for examination and morphologic detection of viruses in a sample within 10 min (84). Based on the unique morphology of different virus families and measurement of the size of the virus particle, EM allows for a virus to be classified into a particular family relatively easily.

Many limitations to EM exist, so that it is usually only available in reference centers. The availability of EM is limited by the cost of the instrument and the need for expertise in reading grids. EM is not well suited for screening large numbers of routine samples, as may be required in a clinical diagnostic laboratory. Estimates on the amount of virus needed in order to be detectable by EM have ranged from 10^5 to 10^8 particles/ml, making it less sensitive than other techniques (84, 85).

NUCLEIC ACID DETECTION

Identification of viral nucleic acids is based on relatively conserved and unique nucleotide sequences which can be

copied into complementary oligonucleotides by polymerases, cut at defined sequence-specific sites by restriction endonucleases, and differentially annealed to complementary sequences under defined conditions. The relatively short length of viral genomes has made viruses ideal candidates for nucleic acid-based diagnosis.

NATs were used to a limited extent before the advent of current amplification assays such as PCR. These consisted of simple hybridization assays such as in the case of the human papillomaviruses (86) as well as detection of defined size segments of viral genome present either endogenously in the case of rotavirus, or resulting from restriction endonuclease digestion as in the case of herpes and adenoviruses (87, 88). With the advent of the PCR assay, NAT-based diagnosis has been extended to nearly all recognized viruses. The PCR assay has evolved from conventional PCR, where products are identified by agarose gel electrophoresis, to real-time PCR, where products are identified using probes or intercalating dyes within the reaction and has now progressed to the development of microarray-based assays either in chip or bead-based formats. Finally, PCR assays have been adapted for quantitation of specific viruses in a specimen.

Polymerase Chain Reaction (PCR) Assay

The development of the PCR assay for DNA virus diagnosis, combined with the preceding reverse transcriptase step for RNA viruses, was a major breakthrough in diagnostic virology. This methodology allows for greatly enhanced levels of viral detection, because for every infectious unit there are numerous defective or otherwise noninfectious particles, all containing the target genome. As shown in Figure 3, the PCR assay is based on cycling a mix of specimen DNA, virus specific oligonucleotide primers in high excess, a thermostable polymerase, and nucleotide triphosphates at a high temperature at which the target DNA strands separate, and a low temperature stage at which the primers anneal to their complementary sites, and an intermediate temperature stage

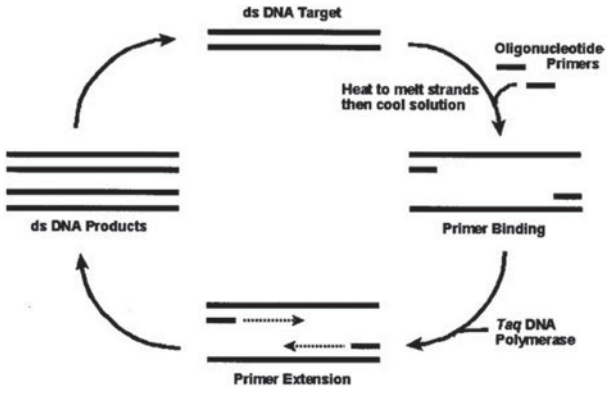

FIGURE 3 Polymerase chain reaction. The strands of the target DNA are separated by heating (melting) and on cooling they anneal with the complementary primers present in excess. The thermostable DNA polymerase extends the primers forming two double-stranded DNA molecules. On subsequent heating, the strands separate and each anneals with the complementary primer. The cycling of temperature between melting, annealing, and primer extension is repeated multiple times and the number of product strands is doubled with each cycle. (Reprinted from reference (185) with permission.)

optimal for the polymerase to elongate the complementary strand as an extension from the primers (89). Each time the cycle is repeated, the number of copies or amplicons doubles such that after 20 cycles, there are potentially over one million amplicons from a single target molecule.

Primers are the major determinant of the sensitivity and specificity of each PCR assay. Since the sequences of most viral genomes are known, at least at the genus level, identifying oligonucleotide primers to conserved regions of the virus genome is feasible. While primers for "in house" assays are known, those for commercial assays may not be readily available. In PCR assays for RNA viruses, a virus-specific primer or a random hexamer primer have been found acceptable for the initial reverse transcriptase reaction to produce the complementary DNA product for amplification. The sensitivity and specificity of these assays is also influenced by the enzymes, buffers, and cycling temperatures and these must be optimized for each specific assay.

Extraction of Nucleic Acid from Specimens

Because of their sensitivity, NATs such as PCR are less fastidious as to the nature of the specimen, such as the need of infectious viruses for isolation, or intact, infected cells for immunofluorescence microscopy. Nucleic acids are generally extracted using commercial kits which are based on the differential binding of the nucleic acid to silica under conditions of different ionic strength. Extracted DNA is stable in EDTA solutions which chelate the magnesium required for DNase activity. RNA, which is less stable, can be preserved using an RNase inhibitor. Likewise, extracted RNAs and DNAs are stable when frozen at −80°C. Automated extractors allow for scaled up extractions of specimens.

Detection of Amplicons

Conventional detection of amplicons has been by agarose gel electrophoresis with staining by ethidium bromide. Other DNA intercalating dyes such as SYBR® Green are an option, as they are less toxic and easier to dispose of but also more expensive. Digestion of the amplicon preparation with restriction endonucleases before electrophoresis enhances the specificity of the assay and allows for more precise identification of the virus at the level of genus and type (90). For enhanced detection, the amplicons resolved on gel electrophoresis can be transferred to a membrane and probed by Southern blotting (91). This can also be accomplished by performing the hybridization of the PCR products in a microtitre plate format which allows for final analysis with a plate reader, an approach utilized in commercial kits.

Multiplex PCR

Multiple viruses can be responsible for the same clinical presentation such as in a respiratory infection. Diagnosis on a single specimen can be performed by a PCR assay that can detect multiple genome targets. Such multiplex assays, though feasible, require careful design to ensure that the primers chosen have similar annealing temperatures, do not react with other primers to reduce the sensitivity, and that the amplicons can readily be detected based on their size or by probes specific for each viral amplicon sequence (92). Multiplexing has been elegantly addressed in recently developed commercial respiratory virus assays. In an example of such an assay, any RNA viral genomes are reverse transcribed to complementary DNA using random primers (93). The preparation is then subjected to a multiplex PCR reaction with primers to all target virus sequences. The PCR products are then subjected to a multiplex target specific

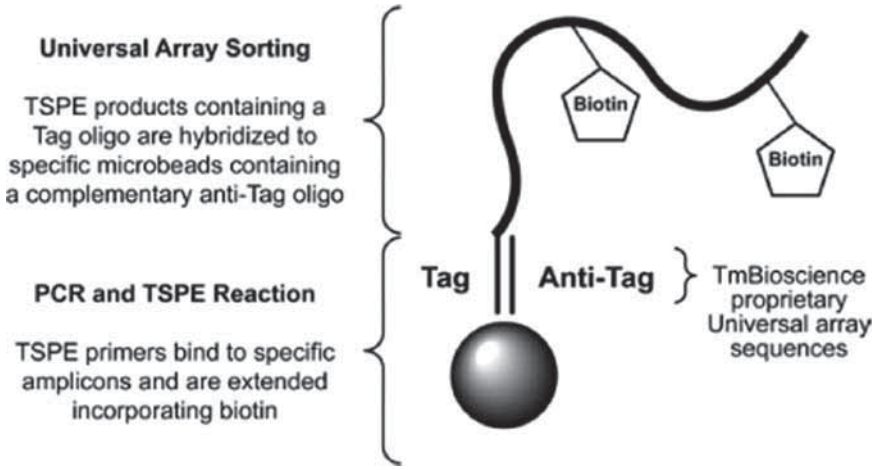

FIGURE 4 The RVP assay (Luminex Corporation, Austin, TX). Identification of the target-specific primer extension reaction that had been captured on the microbead is achieved through the oligonucleotide tag. Sorting of the microbeads occurs in the Luminex 100 flow cell instrument, which identifies colored beads with one laser and a phycoerythrin signal on the attached extended amplicon with a second laser. TSPE: target-specific primer extension.

extension reaction using primers specific for the viral cDNA sequences which also have respective proprietary target-specific oligonucleotide tags at the 5′-end in a reaction mixture that contains biotinylated deoxyribonucleoside triphosphates. The products of this extension reaction are reacted with microbeads with covalently attached anti-tag sequences unique to each virus type to which the complementary tagged sequences hybridize. The preparation is then analyzed by passage through the Luminex flow cell, where the unique signals of each bead type and the common signal from the biotin-streptavidin phycoerythrin conjugate are measured as illustrated in Figure 4.

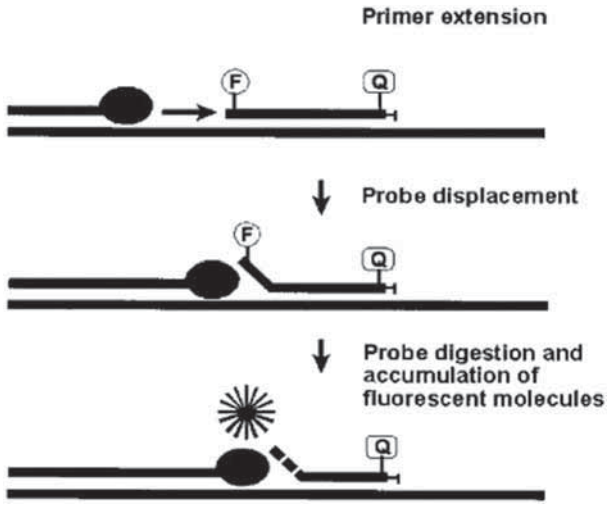

FIGURE 5 Real-time PCR. TaqMan process. In this thermocycling reaction, the internal probe, which is conjugated to the fluorescent dye F and the quencher dye Q hybridizes with the denatured target DNA. When these two dyes are present in close proximity on the probe, the fluorescence of the F dye is quenched. When the new strand being synthesized as an extension of the terminal primers, reaches the probe, it is digested by the 5′-exonuclease activity of the thermostable polymerase liberating the F dye resulting in the generation of a fluorescent signal. (Reprinted from reference (185) with permission.)

Real-Time PCR

Real-time PCR is an enhancement of the conventional PCR reaction in which amplicons are detected in a closed system after each cycle. This initially involved the detection of fluorescence of the SYBR Green dye that had intercalated into the amplicons before each melting step of the cycle (94). Subsequent detection systems incorporate the energy transfer reactions between fluorescent dyes in a process called fluorescence resonance energy transfer (FRET). This consists of 2 dyes one of which is called the donor dye and the other, the acceptor dye. When they are brought close together through the annealing of two adjacent primers to the target, the upstream one labeled at the 3′-end with the donor dye (for example, fluorescein) and the downstream one at the 5′-end with the acceptor dye (for example, cy5), light is absorbed by the donor dye and emitted by the acceptor thereby creating a signal (95).

An alternative configuration is a single probe labeled at the 5′-end by a reporter dye and at the 3′-end by a quencher dye (96). The probe exhibits minimal fluorescence in the unhybridized state when the oligonucleotide is variably folded on itself, but exhibits strong fluorescence at defined wavelengths when the quencher becomes separated from the reporter fluorophore i.e., when the probe is linearized by hybridization to a target sequence. Such structures are called a molecular beacon (97). Alternatively, the reporter fluorophore and quencher, both of which are linked to the probe, may be separated after the probe hybridizes to the amplicon by the 5′-exonuclease action of the thermostable polymerase in the "TaqMan" platform, as illustrated in Figure 5 (98). In this case, the fluorophore accumulates in the reaction mixture in increasing quantities with each cycle.

Real-time PCR has a number of advantages: it is more rapid than conventional PCR; does not include post-amplification processing, thereby minimizing amplicon contamination; and is applicable to quantitative assays. Disadvantages include the inherent difficulty of readily monitoring for amplicon size, which precludes the utilization of digestion with restriction endonucleases as a control,

difficulty of performing nested PCR, and limitations on the size of amplicons and probes. Furthermore, a mutation occurring in the sequence targeted by the probe would preclude the detection of the amplicon in contrast to it still being detected by analysis of the products by gel electrophoresis. Nevertheless, the advantages of speed, sensitivity, and reduction of amplicon contamination have made real-time PCR the platform of choice in diagnostic virology.

Fully Integrated Automated Systems

Integrated systems that perform extraction of the nucleic acid, amplification, and analysis of the findings have been developed. These allow virus diagnosis within a short period of time in a setting of minimal laboratory requirement. Examples of these are the application of the Cepheid GeneXpert to the rapid diagnosis of respiratory viruses and enteroviruses (99, 100).

Non-PCR-Based Nucleic Acid Amplification Systems

Nucleic acid amplification can be performed using approaches other than PCR.

Strand Displacement Amplification

This approach is based on the use of oligonucleotide primers containing a restriction endonuclease cleavage site, a DNA polymerase deficient in 5′-exonuclease activity, the cognate restriction endonuclease, and nucleotide triphosphates of which dCTP has a alpha-thiol group (101). In the reaction, the primer binds to the target sequence on the viral genome, and the polymerase synthesizes a double-stranded product with the endonuclease cleavage site at the 5′-end of the new strand. The restriction endonuclease introduces a nick in the primer site of the new amplicon but not in the complementary strand that has the thiolated dCTP. The nick is recognized by the DNA polymerase which extends the oligonucleotide downstream from the nick and displaces the nicked strand. Annealing of the antisense primer also containing the restriction endonuclease site allows the process to continue on the newly synthesized strand. As shown in Figure 6, this process results in an exponential amplification of the area bracketed by the primers under isothermal conditions. The BDProbeTec (Becton, Dickinson and Company, Franklin Lakes, NJ) is an example of an automated platform utilizing this method. The assay has a sensitivity of 5 to 50 genome copies per ml and requires approximately one hour for completion.

Ligase Chain Reaction

The principle of this assay, illustrated in Figure 7, involves thermal cycling with the polymerase replaced by a thermostable ligase (102). The assay involves the ligation of a pair of primers complementary to the full length of the target sequence of each strand that hybridize at adjacent positions and are joined by the ligase. Increasing the temperature results in the dissociation of the primer-derived strands, to which new primers can anneal at reduced temperature, and allows the reaction to continue with the formation of the ligated primers as the end product. The assay has a sensitivity of 5 to 50 genome copies per ml and is reported to be minimally susceptible to inhibitors in the specimen.

Nucleic Acid Sequence-Based Amplification (NASBA)

This isothermal assay is based on the amplification of predominantly RNA genome targets using 2 primers, one of which has a T7 promoter sequence at the 5′-end, a reverse transcriptase, a T7 DNA-dependent RNA polymerase, and RNase H along with the deoxynucleotide triphosphates (103). In the assay, the primer with the T7 promoter hybridizes to the RNA template and is extended by the reverse transcriptase. The RNA portion of the heteroduplex is digested by RNase H and the reverse primer hybridizes to the new DNA strand and is extended by the reverse transcriptase to produce a double-stranded DNA. The T7 polymerase copies multiple RNA strands off the DNA template in a primer-independent manner. Each of these can bind the primers and repeat the entire process. These reactions are illustrated in Figure 8. A closely related approach, transcription-mediated amplification (TMA), initially called self-sustained sequence replication, is very similar, differing only in that conditions are set so that the endogenous RNase H activity of the reverse transcriptase is able to substitute for the exogenously added RNAse H (104). This platform has been adopted in kits such as those from Gen-Probe, Inc., San Diego, CA and BioMérieux SA, Marcy l'Etoile, France. The assay is reported to have a sensitivity of 5 to 50 genome copies per ml.

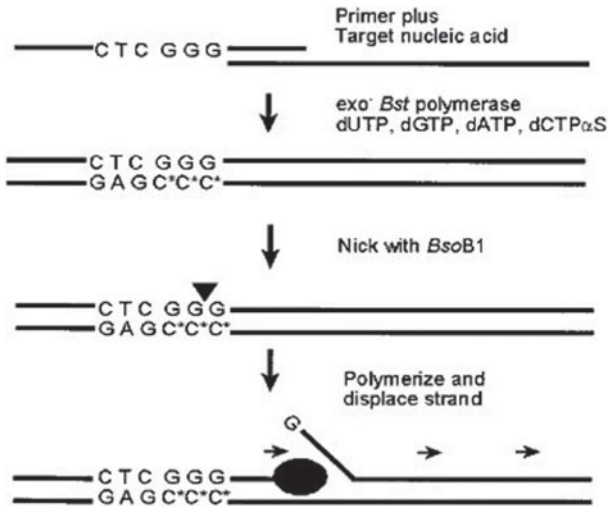

FIGURE 6 Strand displacement amplification. This isothermal amplification assay is based on four requirements namely primers which include an upstream restriction endonuclease site (BsoB1), the cognate enzyme (BsoB1), a DNA polymerase lacking 5′-exonuclease activity and nucleotide triphosphates of which one has been modified to contain an alpha-thiol group (dCTPαS). In this reaction, the target DNA anneals with the primers and is converted to a double-stranded form by the polymerase. The restriction endonuclease introduces a cleavage in the primer sequence and the polymerase synthesizes a new strand from this site and displaces the existing strand. The restriction endonuclease is not able to cut the newly synthesized strands because of the modified nucleotides and can only introduce cuts in the sites present in the primers. Restriction site bearing primers are designed to anneal to sequences of both strands of the target DNA resulting in an exponential synthesis of displaced strands. (Reprinted from reference (185) with permission.)

Recombinase Polymerase Amplification

An intriguing application of the recombinase polymerase reaction using phage T4 proteins UvsX and its cofactor UvsY allows for the primer-based amplification of DNA under isothermal conditions (105). This approach allows

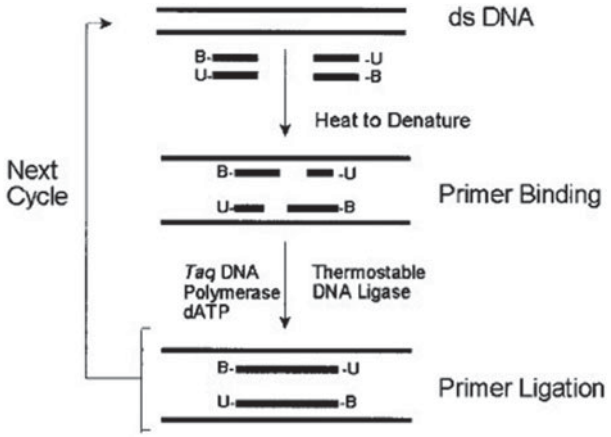

FIGURE 7 Ligase chain reaction. This reaction includes two pairs of primers, each pair annealing to one strand of heat denatured target DNA with a gap of 2 to 7 nucleotides between. The primers are designed to bind so that the gap between them consists of a single nucleotide type. The reaction also contains the relevant nucleotide triphosphates, a thermostable DNA polymerase and a thermostable DNA ligase. Once the gap is filled by the polymerase, the ligase joins the last nucleotide to the downstream primer. The temperature is then raised to denature the product and then lowered to allow further primer binding. The cycle is repeated so additional primer pairs can be ligated. By having the upstream primer labeled at the 5′-end with biotin (B) and the downstream primer at the 3′-end with a fluorescent label, the products can be captured on a solid phase and tested for presence of the fluorescent label. (Reprinted from reference (185) with permission.)

for a rapid diagnosis of viruses under field conditions, as has been shown for dengue virus as well as for MERS-coronavirus and for other biothreat agents (106–108).The assay has been reported to be as sensitive as conventional RT-PCR, with results available in as little as 20 minutes.

Hybridization-Based Assays

Hybridization with labeled probes has long been used for detection of viral genomes. This has been refined to a high sensitivity in the branched-chain DNA assay which is based on signal amplification (109). In this process, shown in Figure 9, the genome is hybridized to a primary probe of complementary sequence which is immobilized on a solid phase. The genome then further hybridizes to secondary probes, which then hybridize with an amplification multimer. The latter hybridizes further with labeled probes that allow for the detection of the branched chain structure. Branched DNA assays have a sensitivity of 50 to 500 genome copies per ml. An alternative approach, exemplified by the hybrid capture assay, involves hybridization in solution between a denatured DNA target and complementary RNA probes resulting in the formation of a DNA-RNA hybrid. This structure is recognized by an antibody which is then detected by an immunoassay detection system. Such an approach has been commercially applied in the Digene Corporation Hybrid Capture assays (Qiagen, Venlo, Netherlands). Hybridization assays, while not as sensitive as nucleic acid amplification (i.e., limit of detection of 500 to 1,000 genome copies per ml), have an ability to quantitate the amount of genome present in the specimen. These have been successfully adapted to the quantitation of hepatitis C virus (HCV) RNA (110).

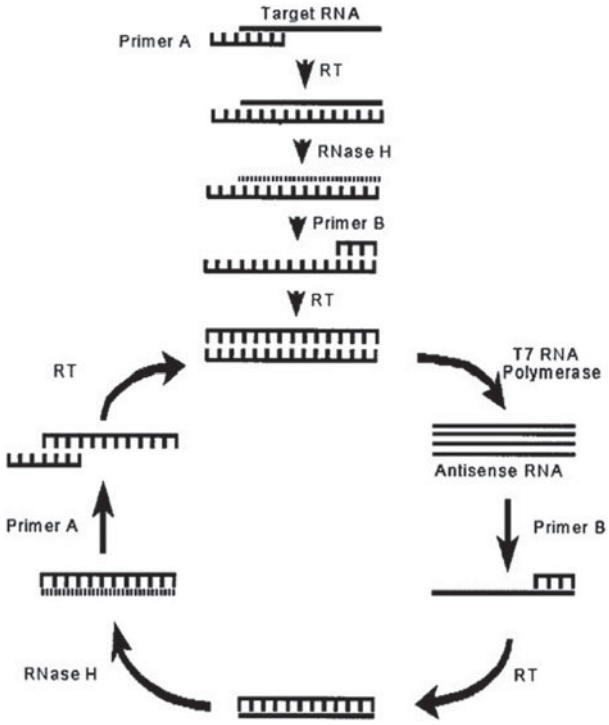

FIGURE 8 Nucleic acid sequence-based amplification (NASBA). This procedure is particularly well adapted to the detection of RNA. The reaction mixture consists of one primer which contains the sequence for the T7 RNA polymerase promoter at its 5′-end, a second primer at the downstream end of the sequence to be amplified, T7 RNA polymerase, reverse transcriptase, and RNAse H as well as the ribo- and deoxyribonucleotide triphosphates. When the primer containing the T7 promoter anneals to the target RNA, the reverse transcriptase synthesizes the complementary DNA strand and the RNA portion of this duplex is digested by the RNAse H. After the binding of the downstream primer, the reverse transcriptase synthesizes a double-stranded DNA with a T7 promoter at one end which serves as a template for the T7 RNA polymerase that synthesizes approximately 1,000 copies of antisense RNA in a promoter-dependent manner. This RNA can be further reverse transcribed to double-stranded DNA which can serve as a template on which the RNA polymerase can synthesize multiple antisense RNA copies. (Reprinted from reference (185) with permission.)

Quantitative Nucleic Acid Tests

Initially, quantitation by PCR assays was accomplished by performing the assay on serial dilutions of the specimen. Currently, quantitative PCR assays contain an internal standard target of defined concentration that serves as a reference on which the calculations of concentrations of the analyte nucleic acid are based. Due to inherent variability, most commercial assays claim an accuracy within 0.5 log10, or approximately 3-fold. These approaches are based on the assumption that the amplification efficiencies of the standard and the target are nearly equivalent. Quantitation can also be achieved by NASBA and branch chain DNA assays (111). The latter being a signal amplification assay is considered less variable but also less sensitive than target amplification assays. The NASBA and PCR assays have been optimized to detect the product in the log phase of the

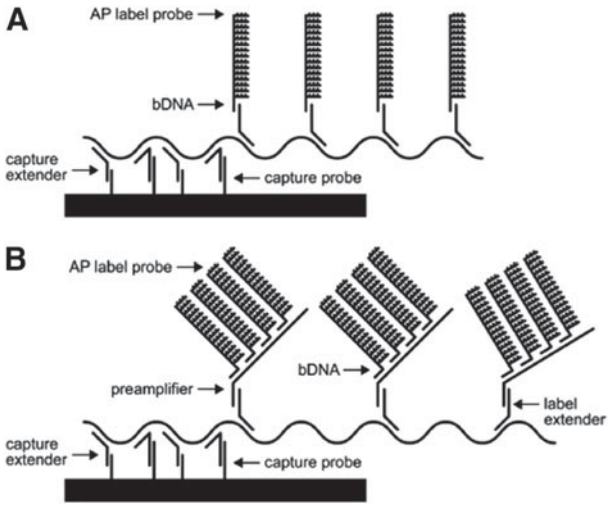

FIGURE 9 Branched chain DNA (bDNA) assay of first (A) and third (B) generations. Target DNA hybridizes to capture probes linked to a solid phase. A short oligonucleotide called the label extender hybridizes with complementary sequences on the target DNA and with either the amplifier oligonucleotide shown in A or a longer oligonucleotide called the preamplifier that contains multiple repeat sequences shown in B. Amplifier oligonucleotides then hybridize to the alkaline phosphatase (AP)-linked probes shown in A as well as the preamplifier sites shown in B. Alkaline phosphatase is detected by standard reagents for the enzyme.

amplification rather than at the end, thereby increasing the dynamic range. To overcome some of the barriers associated with quantitative PCR such as the requirement for standard curves for absolute quantification, and the efficiency dependence of the polymerase reaction which can lead to data variability from sample contaminants, digital PCR (dPCR) was introduced (112). The technique is based on the partitioning of a single sample (containing DNA or RNA, primers, polymerase, and a fluorescent probe) into thousands of individual reaction vessels followed by amplification. Each reactor can then be sorted by fluorescent signal as positive or negative delineating the presence or absence of target (113).

Controls

Controls are of critical importance in NAT assays, because the analytical sensitivity is high and because the amplicons generated in the PCR reactions are relatively stable and serve as a potential source of contamination. Efforts to overcome this problem include the use of assays that do not create DNA amplicons such as NASBA, branched DNA, and real-time PCR which does not involve the opening of the tube with amplified products. The addition of deoxyuridine triphosphate together with uracil DNA glycosylase to the PCR, which destroys the amplicon sequence at the end of the assay (114), is commonly used. Fastidious adherence to the design of the laboratories so that amplicon contamination is avoided by restriction of movement among clean and dirty rooms is essential. Amplicon contamination of the environment can be further reduced by wiping the surfaces with household bleach, exposing working surfaces to short ultraviolet light, and working in laminar flow biological safety cabinets with appropriate clothing and use of gloves.

NATs require numerous controls to ensure that the amplification reactions did occur and that they were free of any contamination. These include amplification controls or target-related oligonucleotides such as genomes of related viruses with sequences complementary to the same probes, genomes of other viruses for which probes are provided, and DNA of defined sequences in plasmid vectors or RNA produced by T7 polymerase from a recombinant DNA target with T7 promoter. It is particularly important to protect the RNA controls from endogenous RNases by inclusion of RNase inhibitors, or by encapsidating them into proteins such as that of MS1 phage as in the case of "armoured RNA" (115). Controls are also added to the specimen or aliquot thereof before extraction to serve as extraction controls. Water, free of any target, is an essential negative control. Finally, a sample quality control consisting of a common gene such as Beta-globin or RNase P helps to validate that the specimen contained adequate tissue for testing.

Application of Nucleic Acid Testing in Virology

The very high sensitivity of NATs has greatly improved the ability to obtain an accurate viral diagnosis, but has also created a need for enhanced insight into the interpretation of the test results and the need to more thoroughly understand the pathogenesis of viral infections. Applications of NATs include detection of the virus in the patient specimen in acute and chronic infections, the potential to subtype the virus to the level of its genome sequence, to quantitate the concentration of the virus to determine the extent of the infection, the likelihood for transmission, and the response to antiviral therapy, and to monitor the development of strains resistant to antiviral agents.

Routine Diagnosis of Virus Infection

Detection of most common viruses can be performed by NAT within comparable or superior turnaround times, and with substantially increased sensitivity, relative to conventional methods. Using real-time PCR, most of these viruses can be identified in patient specimens on a same-day to next-day basis, making the diagnosis relevant for patient management. In addition, the development of multiplex assays has allowed for an unprecedented breadth of testing for all known respiratory viruses in a patient specimen within a working day using a single test (93). Likewise, nucleic acid microarray technology is believed to have a similar potential (116).

Real-time PCR has been successfully applied to same-day testing for herpes simplex virus either as "in-house" or commercially marketed assays (117). The diagnosis of noroviruses and other gastrointestinal viruses by NAT is more efficient and reliable than ELISA or EM (118). While enteroviruses have conventionally been diagnosed by isolation in cell culture, NAT is substantially more sensitive, an important consideration in the diagnosis of neurotropic viruses (119). Accordingly, laboratories are becoming increasingly reliant on NAT for routine virus diagnosis, a trend that is likely to continue as the costs of these tests decrease with increased volumes of use, and the implementation of further automation.

Diagnosis of Novel and Emerging Viral Infections

NAT is particularly suitable for the diagnosis of novel and emerging infections. The ones that stand out in recent times are the diagnosis of the Sin Nombre hantavirus in 1993, the diagnosis of SARS-coronavirus in 2003, and the diagnosis of MERS-coronavirus more recently (120–122). A major

advantage of NAT in the workup for novel agents of as yet unknown risk, or those associated with high morbidity and mortality, is that much of the investigation does not need to be performed under BSL-3 conditions. The risk of working with extracted nucleic acids is minimal under BSL-2 conditions. Group-specific PCR assays based on conserved sequences can provide evidence as to the identity of the virus at the family level, and the virus is further characterized by the sequencing of the amplicons. Similarly, amplicons from randomly primed PCR reactions can be characterized by shotgun sequencing. In implementing tests for pandemic influenza in 2009, NAT reagents were readily available, the primer sequences quickly identified and communicated to laboratories, the extraction and NAT assays were readily scaled up 10-fold, and testing was performed in BSL-2 laboratories with specimen extraction being the only risky step (123).

Diagnosis of Chronic Viral Infections

Primary diagnosis of chronic viral infections such as HIV, HBV, and HCV is generally accomplished by serology, but NAT has an important role in the follow-up of these infections. With NAT, one can differentiate between a chronic HCV infection which is present in approximately 75% of seropositive patients, and successful resolution of the infection in the remainder (124). NAT is used to determine whether a child, born of an HIV seropositive mother, is infected or whether the antibody in his/her serum comes from passive maternal transfer (125). NAT can help resolve conundrums in HBV serology and establish or rule out infectivity. Likewise, NAT is used for testing blood donors to rule out the use of the very rare infected unit that cannot be identified by serology and other screening methods. Finally, NAT is often the only realistic test to use to investigate infection in immunocompromised patients unable to mount a humoral immune response.

NATs that can assess the concentration of the virus in the blood have proven valuable as the viral load correlates with the risk of transmission. Pregnant mothers with HIV are treated with antiviral drugs to suppress the viral load, which can be monitored (125). Likewise, the viral load correlates directly with the risk of transmission in HIV-discordant couples (126). Finally, for healthcare workers with chronic HBV infections, a high viral load can be used to exclude them from certain procedures.

Viral Diagnosis in the Management of Immunocompromised Patients

Patients immunocompromised either through infection, malignancy, or iatrogenically, such as those having undergone transplants, are subject to many life-threatening viral infections. Infections of particular concern to such patients are those of the herpesviruses, EBV and CMV (127). Monitoring the viral load in the blood of transplant patients, especially those in the pediatric group, and either modulating immunosuppression or using antiviral drugs such as ganciclovir is critical for their survival. Likewise, monitoring the presence of adenoviruses and the human polyomavirus BK in the blood and urine are important in transplant patients and these are most commonly done by NAT (78, 128).

Monitoring Response to Antiviral Therapy and Antiviral Drug Resistance

In HCV-infected individuals, the duration of antiviral therapy with interferon and ribavirin has been determined by the genotype of the virus and the efficacy of the treatment on the viremia (129). The viral genotype is obtained by characterizing the amplified nontranslated 5′-region of the genome by either sequence analysis or by hybridization to probes immobilized as lines on a solid phase (Line blot). The response to treatment of specific genotypes is monitored by quantitative assays such as branched chain DNA and quantitative PCR. These are vital assays for HCV treatment, the utilization of which is dependent on a brisk fall in viremia and the absence of detectable virus at the end of treatment. Likewise, HBV viral load can be quantitated by branched chain DNA or PCR assays and used to monitor the response to antiviral drugs.

The capacity to accurately assess the viral load is essential in the management of HIV infections, where there is a relationship between viral load, the CD4 cell count, and disease progression (130). When patients are undergoing antiviral treatment, the goal is to maintain the viral load at levels below 50 genome copies per ml of plasma, the lower limit of detection of quantitative assays (131). In another application, an increased viral load in a patient receiving antiviral therapy may suggest the development of viral resistance, which can be confirmed by specific phenotypic or genotypic resistance assays.

Nucleic Acid Sequencing

DNA sequencing has evolved from a research tool to become an expected part of diagnostic virology. It has proven its value by greatly simplifying the typing of enteroviruses and adenoviruses (132, 133). It allows for identifying specific genotypes of noroviruses and rotaviruses (118, 134) and for monitoring the drift of influenza A viruses at the nucleotide level over time (135). Newer sequencing technologies such as pyrosequencing and shotgun sequencing allow for even more effective application of sequencing to diagnostics, and are indicative of the future directions of these technologies in viral diagnosis (136). This includes more accurate determination of quasispecies of the virus present in a clinical specimen. Finally, high throughput sequencing allows for metagenomics analysis of the nucleic acids present in clinical specimens to comprehensively identify existing and potentially novel viruses (137, 138).

SERODIAGNOSIS OF VIRAL DISEASES

Serologic assays for the detection of antibodies directed against viruses continue to play a major role in the clinical laboratory. For individual patients, these assays can be used for diagnosis of acute or chronic infections, determination of immune status (either as a result of natural infection or immunization), and for detection of individuals latently infected with certain viruses (e.g., *Herpesviridae*) (Table 7). Clinically, these assays are used for seroepidemiologic surveys, donor screening for prevention of transmission of blood-borne viruses (e.g., blood, organ, tissue, etc.) from donor to recipient, preemployment of healthcare workers and others, infection control purposes, prenatal screening of mothers for determining risk of vertical transmission and planning of preventive strategies (e.g., hepatitis B), and for managing risks associated with occupational or other exposures (e.g., needlestick injuries, etc.). In addition to preventing transmission of blood-borne viruses during transplantation or transfusion, knowing the serologic status of organ donors and recipients prior to transplantation for certain viruses such as CMV is important when considering the type of donor and blood products to be given, and in

TABLE 7 Utility of serological determinations in clinical virology

Application	Most common virus(es)[a]
Diagnosis of recent or chronic infections	
Hepatitis	Hepatitis viruses A-E, CMV, EBV, HSV, VZV, HIV, coxsackievirus B, adenovirus, yellow fever virus
Central nervous system	HSV, CMV, VZV, EBV, HHV-6, enteroviruses, West Nile virus and other arboviruses, measles virus, mumps virus, rubella virus, rabies virus, HIV, LCV
Congenital/perinatal	CMV, HSV, VZV, rubella virus, HBV, HCV, parvovirus B19, LCV
Exanthems	Measles virus, rubella virus, parvovirus B19, HHV-6, HHV-7, arboviruses
Myocarditis/pericarditis	Coxsackievirus B types 1–5, influenza virus types A and B, CMV
Infectious mononucleosis	
Heterophile antibody positive	EBV
Heterophile antibody negative	EBV[b], CMV, HIV, rubella virus, HHV-6
Nonspecific febrile illness	CMV, EBV, HHV-6, HHV-7, Parvovirus B19, HIV, dengue virus, Colorado tick fever virus
T-cell leukemia	HTLV-I/II
Hemorrhagic fever	Filoviruses, arenaviruses, flaviviruses, bunyaviruses
Hantavirus pulmonary syndrome	Sin Nombre virus, other hantaviruses
Screening for immune status[c]	
Preemployment	VZV, rubella virus, measles virus, HBV
Prenatal	Rubella virus, CMV, HSV, VZV, Parvovirus B19, HBV, HCV, HIV
Pretransplant	CMV, HSV, EBV, VZV, HBV, HCV, HIV
Blood donation	HIV, HBV, HCV, HTLV-I/II
Postexposure	HIV, HAV, HBV, HCV, VZV
Epidemiology and surveillance	All viruses
Verify response to vaccination	HAV, HBV, HPV, VZV, measles virus, mumps virus, rubella virus

[a]CMV, cytomegalovirus; EBV, Epstein-Barr virus; HSV, herpes simplex virus; VZV, varicella-zoster virus; HIV, human immunodeficiency virus; LCV, lymphocytic choriomeningitis virus; HAV, hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HHV-6, human herpes virus-6; HHV-7, human herpes virus-7; HPV, human papillomavirus; HTLV-I/II, human T-lymphotropic virus I and II; WNV, West Nile virus.

[b]A comprehensive panel of EBV-specific serological tests should be performed for patients with heterophile-negative infectious mononucleosis, since only 60% of individuals will have heterophile antibodies by the second week of EBV mononucleosis and the test is usually negative in children ≤ 4 years of age. Commercial EIA and IFA EBV serological tests allow for the simultaneous titration of virus-specific antibodies to the viral capsid, early and nuclear antigens of the virus. Interpretation of the panel permits identification of current or recent primary infection, recurrent or chronic infection, or past infection with EBV.

[c]Determination of immune status either as a result of natural infection or immunization.

determining the treatment or prophylaxis to be used following transplantation.

Detection of virus-specific antibodies in a patient's serum or plasma may also be the only means of making a viral diagnosis under certain circumstances. A number of viruses are impossible, difficult, or even hazardous to grow in culture, or difficult to detect by other methods. Proper specimens for culture or direct detection assays may be difficult to obtain or may not be obtained because viremia or virus replication and shedding have subsided to undetectable levels when symptoms occur. In these situations, serodiagnosis is often helpful in confirming a recent or past infection with a specific virus. Sometimes, the clinical significance of detecting a virus by means of a direct assay or by molecular techniques may be uncertain. In these situations, serology may assist in establishing a causal relationship (e.g., significant changes in antibody titre to the detected virus or presence of IgM antibodies to a specific virus). Serology is also an invaluable tool in the public health domain, whether to assess incidence and prevalence of viral diseases for epidemiological studies, surveillance systems, or evaluation of the efficacy of prevention and control programs.

Although traditional methods of viral serodiagnosis are still in use, automated technologies have now become the mainstream of serological testing in clinical laboratories. Use of recombinant antigens and synthetic peptides representing immunodominant regions has improved test sensitivity and specificity for detecting and confirming virus-specific antibodies. At the same time, simple-to-use commercial tests or devices capable of rapidly analyzing single specimens, such as finger prick blood samples, have been introduced for rapid point-of-care testing.

Antibody Response to Viral Infections

Immunoglobulins as markers of humoral response are more useful and reliable than cell-mediated immunity for diagnostic purposes. Antibodies can be detected and measured as they develop in response to an infecting virus or to immunization (Figure 10). Because of the transient nature of the IgM antibody response, its presence is generally indicative of current or recent viral infection. However, reactivation of latent or chronic viral infections (e.g., HSV, Hepatitis B, others) can result in detectable levels of IgM antibodies, making the distinction between acute infection and reactivation disease difficult. As well, certain viruses such as EBV can result in a polyclonal B-cell stimulation with elevated IgM antibodies to multiple different viruses. A significant (classically a 4-fold) rise in IgG antibody concentration between acute and convalescent serum samples collected 10 to 14 days apart is also accepted as evidence of an acute or

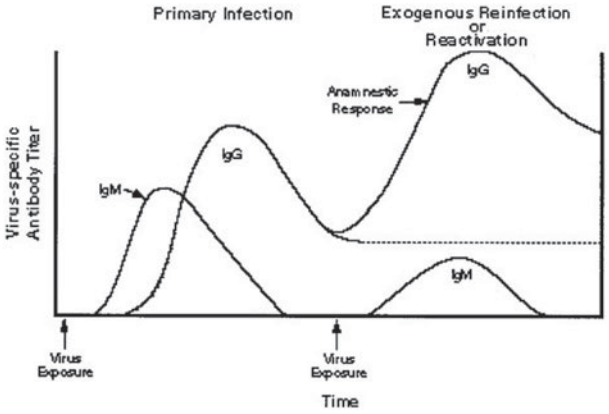

FIGURE 10 Diagram depicting the typical IgM and IgG antibody responses following primary viral infection, reactivation or reinfection. During primary infection, IgM appears within several days after onset of symptoms, peaks at 7 to 10 days, and normally declines to undetectable levels within 1 to 3 months. Following natural viral infection or after successful immunization, IgG antibodies appear several days after the production of IgM, reaches higher levels than IgM, and can persist for years, even life-long, in lower quantities. During reactivation or exogenous reinfection, an anamnestic response in IgG antibodies will occur and an IgM response may or may not be observed.

recent infection. In some instances, measurement of antibody avidity may help in distinguishing a recent infection from a past infection with associated reactivation (139, 140). Avidity refers to the accumulated strength of multiple affinities between an antibody and antigen as a result of multiple antigen-binding sites simultaneously interacting with the target antigenic epitopes. As the antibody response to a viral infection matures over time, avidity of the antibody–antigen interaction increases. Hence, low avidity is likely to point to a recent primary infection (141). Avidity can only be measured in individuals with detectable IgG antibodies to the particular virus of interest. Avidity tests for rubella virus, measles virus, *Toxoplasma gondii*, CMV, VZV, HIV, hepatitis viruses, EBV, and others have been developed. Although maturation of the IgG antibody response generally occurs within 6 months following infection, in some individuals this may take longer resulting in a prolonged low or intermediate avidity value and a potentially misleading interpretation as evidence of a recent infection (142).

Avidity assays are generally based on direct ELISA methods in which the serum sample is tested in duplicate. In one well, the ELISA is performed as described above while in the other well, a chaotropic agent such as urea capable of breaking antibody–antigen bonds is added after the antigen–antibody reaction is allowed to occur, but before the detector antibody is added. A comparison is then made between the reading of the two wells and a result is generated which is reported as an avidity index (AI). A low AI suggests relatively recent infection, whereas a high AI is consistent with a mature or past infection. One of the most common areas where measurement of IgG avidity has been applied is in evaluating pregnant women for possible recent CMV, rubella, and VZV infection.

The role, onset, level, and duration of IgA, IgD, and IgE antibodies are less predictable than either IgM or IgG, and serological tests for these isotypes are generally not performed routinely in diagnostic laboratories.

Specimens for Serodiagnosis

Serum is the specimen of choice for most serological assays; plasma can be used in some instances but is not suitable for all antibody tests unless specified by the manufacturer of the assay. Although antibody tests can be applied to other specimen types (e.g., CSF and other body fluids), most assays have not been validated for use with these specimens and results must be interpreted with caution. In adults, 4 to 7 ml of blood obtained from venipuncture in a collection tube containing no additive or preservative is usually sufficient for most testing. The laboratory will accept smaller volumes of blood, particularly from infants and young children or when few tests are being requested. Whole blood collected and dried on filter paper has been studied as a practical and effective substitution to obtaining serum by venipuncture, especially when screening for antibody to HIV (143). A consumer-controlled home collection kit using whole blood has been licensed for HIV-1 testing. Dried blood specimens have also been used for HBV, HCV, measles, mumps, and rubella antibody screening either because phlebotomy was resisted by the patient or transport and long-term storage of serum and plasma specimens was logistically difficult (144, 145).

Whole blood should be allowed to clot for 30 to 60 minutes at room temperature. A centrifugation step allows separation of the serum from the remainder of the blood components. Collected whole blood should not be frozen as this may cause hemolysis rendering the specimen unusable for serologic testing. Icteric, lipemic, or heat-inactivated sera may also cause erroneous test results and should be avoided, if possible. Serum should be refrigerated at 4°C shortly after separation from the blood clot and during transport to the laboratory. If an extended delay in transport or testing of a specimen (beyond 5 to 7 days) is anticipated (e.g., holding an acute-phase serum until the convalescent-phase serum is collected), freezing the serum to at least −20°C is advised.

A single serum specimen is required to determine the immune status of an individual or to detect IgM-specific antibodies. With few exceptions (e.g., EBV mononucleosis), paired serum specimens, collected 10 to 14 days apart, are required for the diagnosis of acute or recent viral infections when specimens are tested for IgG antibodies alone. The acute-phase serum should be obtained as soon as possible during the course of the illness, and no later than 5 to 7 days after onset. The most useful results are obtained by testing acute- and convalescent-phase sera simultaneously in a single assay run. Evaluation of serum for antibodies to so-called TORCH (*Toxoplasma gondii*, rubella virus, CMV, and HSV) agents associated with congenital and peripartum infections requires that two serum specimens be submitted for testing: one from the mother and the other from her infant. To identify congenital infection, the newborn should be younger than 3 weeks when the specimen is obtained. IgG antibodies to a particular virus in the newborn's blood may reflect passive transfer of maternal antibodies *in utero* and may persist for months after birth, making diagnosis of congenital or perinatal infection based on detection of IgG antibodies alone difficult. However, because IgM antibodies do not cross the placenta, their presence in the newborn's blood is consistent with either congenital or perinatal transmission.

CSF may be tested for viral antibodies in patients with viral central nervous system (CNS) disease and may be

superior to NAT detection for some infections (e.g., West Nile virus). For members of the *Herpesviridae* family, and some respiratory viruses that may cause neurologic disease, both CSF and serum specimens should be collected and paired for accurate measurement of virus-specific intrathecal antibody synthesis (146). Simply finding virus-specific IgG antibodies to these agents in the CSF is not diagnostic of CNS infection, because of the expected passage of immunoglobulin (Ig) from blood to CSF. The normal ratio of Ig in blood to CSF is approximately 250:1.

Whole saliva, oral mucosal transudates rich in gingival crevicular fluid, and urine also have been evaluated as noninvasive alternatives to the collection of blood for the detection of antibodies to a number of different viruses (147). Particular attention has been paid to the value of oral fluids and urine for the diagnosis of infections with HIV (148–150). Accuracy of saliva for HIV antibody detection has been shown to be equivalent to serum for clinical usage as well as epidemiological surveillance (147). The sensitivity and specificity of urine tests are inferior to those of blood and oral fluid tests. FDA-approved HIV oral fluid and urine tests are commercially available. Accordingly, several commercial devices have been developed for the collection of oral mucosal transudate specimens. The devices provide a homogeneous specimen rich in plasma-derived IgG and IgM that is passively transferred to the mouth across the mucosa and through the gingival crevices (151). Lastly, vitreous humor has been used for the detection of antibodies to HSV or VZV in individuals with eye infections due to these agents (152, 153).

Procedures for Detecting Viral Antibodies

A variety of methods are available for serodiagnosis of viral infections (reviewed in (67, 154, 155)). The more traditional assays include complement fixation (CF), hemagglutination inhibition (HI), neutralization (NT), immune adherence hemagglutination (IAHA), and indirect and anticomplement (ACIF) immunofluorescence (IFA). With the exception of some commercial IFA kits, these are in-house assays and are rarely performed outside of specialized reference or public health laboratories. Most clinical laboratories use commercially manufactured assays including SPIA, passive latex agglutination (PLA), immunoblotting (IB), and immunochromatographic (IC) tests. The selection of which test method(s) to perform will depend on the patient population and clinical situation, the number of specimens to be tested, turnaround time, ease of testing, and the resources and capabilities of the individual laboratory. Critical clinical information such as the date of onset of disease symptoms, recent travel history, immunization, or medical antecedents may also influence the choice of an adequate analytical strategy. Qualitative measurements of virus-induced antibody can be performed when it is useful to know simply that a specific antibody is present or absent. Quantitation is essential when it is important to know the amount of antibody present, particularly when measuring virus-specific IgG antibodies (e.g., immunity to hepatitis B and rubella viruses).

Complement Fixation

Complement fixation can be used for measuring antibodies against virtually any cultivable virus and it has the distinct advantage of assessing significant rises in antibody levels during acute viral infections (156). The CF method uses the cytolytic property of the complement system as an indirect marker for the antigen–antibody reaction. When virus-specific antibody is present in a patient's serum, it will complex with its corresponding antigen and fix guinea pig complement, thereby preventing lysis of sheep red blood cells (RBC) complexed with anti-erythrocyte antibodies. Conversely, absence of antibody results in activation of the complement cascade by the addition of the sensitized RBCs, thus resulting in their lysis. Although inexpensive in material and reagents, the method is technically demanding, requires rigid standardization and titration of reagents, has a long turnaround time, and acute and convalescent serum must be tested in the same assay run. The CF test is also less sensitive than other methods and cannot be used to determine immune status. Invalid results can occur with sera possessing anticomplementary activity due to nonspecific binding of serum components to the complement used in the assay. For these reasons, the CF test is rarely if ever used today in a routine clinical laboratory.

Hemagglutination Inhibition

HI has been applied to the detection of antibodies to viruses possessing surface proteins that can agglutinate RBCs (157), particularly for the detection of antibodies to influenza, parainfluenza, measles, mumps, and rubella viruses and sometimes to the arboviruses, adenoviruses, and polyomaviruses. Antiviral antibodies in a patient's serum can inhibit the agglutination of RBCs by a viral antigen. The assay is prone to many of the same limitations as CF. In addition, nonspecific inhibitors and natural agglutinins need to be removed from some serum specimens before virus-specific antibodies can be detected. The specificity of HI also varies with the virus, being highly specific for influenza and parainfluenza viruses but less so for arboviruses. Detection of hemagglutinating antibodies is the current standard for assessing the immunogenicity of seasonal influenza vaccines and is often used for retrospective diagnosis of individual infections. The HI assay is also commonly used for subtyping and antigenic characterization of influenza virus isolates by reference laboratories.

The monospot test for the diagnosis of acute/recent EBV infection is a hemagglutination test that relies on the agglutination of horse RBCs by heterophile antibodies in the patient's serum (158). The test is specific for heterophile antibodies produced by the human immune system in response to EBV infection (159). In performing the monospot test, a drop of the patient's serum is mixed on a glass slide with a suspension of guinea-pig kidney stroma and a suspension of beef red cell stroma. A drop of horse RBC suspension is then added to each mixture. After approximately a minute, the slides are examined for the presence or absence of red cell agglutination. If agglutination is stronger with the guinea-pig kidney mixture, the test is positive. If it is stronger with the beef red cell mixture, then the test is negative. Commercial kits for performing the monospot test are available and continue to be widely used because of their relative ease to perform, rapid results, and low cost. The specificity of the test approaches 100% while the sensitivity ranges from 70 to 90%.

Neutralization

Unlike SPIAs, which detect binding antibodies to viral antigens, NT assays detect functional virus-specific antibodies capable of neutralizing or blocking the infectivity and replication of a given virus within a cell culture system (160). The assay can be performed in microtitre plates containing cell monolayers infected with approximately 100 infectious units of virus and a serially diluted antiserum. For greater accuracy, it can also be performed as a Plaque

Reduction Neutralization Test (PRNT). Neutralization titer is usually expressed as the reciprocal of the highest serum dilution resulting in a 90% reduction in the number of infected foci or plaques produced by a virus on a cell monolayer. Detection of neutralizing antibodies is clinically important in establishing protective immunity in response to a viral infection or vaccination. However, the assay is cumbersome, expensive, time-consuming, and in the case of highly pathogenic viruses, needs to be performed under high biosafety conditions (e.g., BSL-3 for West Nile virus and avian influenza A [H5N1] virus). Similar to HI, the quantity of virus used in the system must be carefully determined to obtain accurate results. The NT assay remains the method of choice for the detection of antibodies to the enteroviruses and the most specific test for confirmation of West Nile virus and other arthropod-borne flaviviruses.

A variant of the PRNT is used for the measurement of neutralizing antibodies to rabies virus (161). The Rabies Fluorescent Focus Inhibition Test (RFFIT) remains the gold standard for detection of rabies neutralizing antibodies in a patient's serum. Rather than count plaques, immunofluorescence is used to assess changes in rabies virus replication in cell culture. RFFIT is not generally used for diagnosis of rabies infection. Its major use is for the measurement of rabies neutralizing antibodies in persons vaccinated with the rabies vaccine and whose antibody levels are being monitored routinely due to occupational exposure to rabies virus.

Immune Adherence Hemagglutination

The IAHA method uses the hemagglutinating property of the C3b fragment of complement following attachment to C3b receptors on human type O RBCs as an indirect marker for the antigen–antibody reaction (162). Virus-specific antibodies in serum are detected when complement binds to antibody–antigen complexes that have formed rather than to RBCs, thus inhibiting hemagglutination. The assay is simple, and has a sensitivity and specificity that is better than CF, comparable to IFA, and only slightly less sensitive than SPIA. IAHA is reliable for immune status testing and quantitative measurements of antibody titers. IgM can be detected after fractionation from IgG antibodies. Special attention has to be given to finding an adequate supply of human type O erythrocytes, and to qualify commercial antigen preparations. A prozone effect may occur in the presence of excess virus-specific antibody in the serum sample. IAHA has been used for the detection of antibodies to many different viruses (e.g., rabies, hepatitis, and VZV). It is rarely used in clinical virology laboratories.

Immunofluorescence Assays

IFA are very useful and inexpensive methods that offer the advantages of speed and simplicity for the qualitative and quantitative detection of both IgM or IgG antiviral antibodies in clinical specimens (163). Commercial kits or antigen-coated slides are readily available for many of the common viruses, and labeled secondary antibodies can be purchased separately. Antibody is usually detected by either an indirect IFA or ACIF. In the indirect IFA, dilutions of serum are incubated with virus-infected cells that have been fixed to a glass microscope slide; specific antibody–antigen complexes that form are then detected using an anti-human antibody conjugated with a fluorochrome, most often FITC. A variation of this procedure called monoclonal antibody-enhanced IFA (MIFA) has been designed to increase the sensitivity of the fluorescent signal. It is a three step procedure using an anti-human immunoglobulin mouse monoclonal antibody which is allowed to react with antibody–antigen complexes before an anti-mouse labeled antibody is used in the detection step (164). ACIF differs in that following incubation of test serum with virus-infected cells on a glass slide, fresh complement is added and bound by any specific antigen–antibody complexes that have formed. A fluorescein-labeled anticomplement antibody is then added and binds to the C3 component of complement. ACIF amplifies the fluorescence signal, allowing for the detection of small amounts of antibody or antibodies of low avidity. This method is routinely used to detect antibodies to the nuclear antigen of EBV.

The disadvantages of IFA are that it requires a fluorescence microscope and dark room for examining slides and extensive training and critical evaluation are needed to read and reliably interpret the test results. The number of positively fluorescing cells, as well as the quality and intensity of the fluorescence, must be carefully examined and compared to those of cells reacted with positive and negative control sera. Most manufacturers of commercial kits provide antigen slides in which only 20 to 40% of the cells express viral antigens. Therefore, nonspecific binding of antibodies to the cells is easily discerned since the staining produced by these antibodies involves all of the cells.

Solid-Phase Immunoassays

The term SPIA encompasses a large variety of methods and platforms aimed at detecting immobilized antigen–antibody complexes by means of a reporter signal. They have become the methods of choice for the detection of many virus-specific antibodies because of their speed, convenience, relative simplicity, and excellent sensitivity and specificity. The assay format is quite versatile, has evolved from polypropylene test tubes to microscopic spheres, and is applicable to many viruses and large numbers of specimens at a relatively low cost. As noted earlier, ELISA and EIA are the most popular SPIA used in clinical virology laboratories; they offer the advantages of using highly standardized and stable immunoreagents, especially those using recombinant antigens and synthetic peptides. EIA kits that detect IgG or IgM antibodies to a number of different viruses are available from a variety of commercial sources. A newer generation of assays even provides simultaneous detection of antigen and antibody, thereby improving the early detection of HIV and HCV (165, 166).

As for the detection of viral antigens, both noncompetitive and competitive assays are available, and the results can be evaluated either qualitatively or quantitatively (67). The basic principle of the noncompetitive EIA for the detection of antibodies is similar to that for the detection of viral antigens, except that viral antigen is immobilized on a solid phase and is used to capture free virus-specific antibody in a clinical specimen. The captured antibodies are then detected with the addition of an enzyme-labeled anti-human antibody followed by the addition of a chromogenic substrate to produce a color change. The intensity of the color generated is measured in a spectrophotometer and is usually proportional to the amount of virus-specific antibody in the specimen. Quantification of antibody content can be calculated from a standard curve established from serial dilutions of a reference antibody. In addition to color production, other detector labels have been used including fluorochrome labels such as fluorescein, rhodamine, and Texas red, as well as chemiluminescent substrates.

In a competitive EIA, enzyme-labeled antiviral antibody is mixed with test serum, and the presence of virus-specific

antibodies in the specimen will compete with the labeled antibody for a limited number of viral antigen binding sites on the solid phase. The activity of labeled antibody activity is then measured as described above. The decrease in detectable labeled antibody is inversely proportional to the quantity of antibody present in the sample. Competitive antibody assays are often used to provide greater specificity than noncompetitive assays.

RIAs using radioactive iodine as a label were developed in the 1970s (167). RIAs are less appealing because of the precautions and regulations needed to work with radioisotopes and dispose of these hazardous materials. Consequently, RIA has been largely replaced by EIA.

Microbead-based liquid arrays combine SPIA with flow cytometry to allow simultaneous detection of multiple antibodies (168, 169). The system uses 5.6 μm polystyrene microspheres, each containing a unique spectral address determined by varying the concentrations of red and infrared fluorochromes. Each bead, coated with carboxyl groups, is covalently linked to peptides or proteins. Antiviral antibodies are then allowed to bind to their respective target antigens labeled with a fluorescent reporter molecule. Detection is performed by means of a flow cytometer composed of a dual beam laser detection system. A microfluidic device drives the microspheres into the laser beams. A red laser classifies the bead based on its internal color corresponding to a given antigen. A green laser measures fluorescence intensity corresponding to the reaction of antibodies with the antigen-reporter molecule complex. Microsphere immunoassays can detect up to 100 different viral antigens in a single well in less than 30 s per well, and require only small sample volumes. They have been applied successfully to the detection of antibodies to HIV, respiratory viruses, arboviruses, EBV, and HPV (170–174).

A number of semi-automated and fully automated immunoassay analyzers for SPIA are now commercially available. These systems have found particular utility in the area of blood banking and clinical virology, where extensive test menus now exist for the automated performance of serological assays for antibodies to HIV, hepatitis viruses, viral agents causing congenital infection, and other viruses of clinical importance. The majority of the automated immunoassay analyzers provide random access, walk-away simplicity to perform assays from sample processing through interpretation of results.

Passive Latex Agglutination Assays

PLA is an uncomplicated, convenient, and inexpensive method that is best suited for testing small numbers of specimens. A suspension of latex particles coated with viral antigens is mixed with a clinical specimen and allowed to incubate with rotation for a short time (175). The antigen-coated particles will clump and produce visible agglutination in the presence of virus-specific antibody, which can be visualized with the naked eye. The test can be completed within 10 min and requires limited equipment and technical ability. Both IgG and IgM antibodies are detected without differentiation, and the sensitivity and specificity of PLA are reported to be comparable to those observed for EIA and IFA. However, reading an agglutination reaction by PLA can be subjective, and the results may be difficult to interpret. Also, a prozone or reduction in the degree of agglutination can occur with sera that have high levels of specific antibody, resulting in the need to dilute negative specimens and repeat the assay. PLA is best suited for qualitative determinations of viral antibody, but quantitation is also possible. An automated particle counting technology has been developed which allows objective reading of the agglutination reaction (176). When RBCs are coated with viral antigen, the procedure described for PLA is termed passive hemagglutination.

Immunoblot Assays

IB is a distinct type of SPIA using nitrocellulose or polyvinylidene fluoride membranes as the physical support for the detection of antibodies to specific viral antigens (177). The classical example is the Western blot, an assay widely used for confirmation of positive HIV antibody results obtained by a screening test. In the Western blot assay, whole virus lysates of inactivated and disrupted viral proteins are separated by electrophoresis according to their molecular weight or relative mobility as they migrate through a polyacrylamide gel in the presence of sodium dodecyl sulfate. The resolved protein bands are then transferred onto a membrane. The membrane is then cut into strips. Each strip can then be incubated with its respective serum. If virus-specific antibodies are present in the serum, binding of specific antibodies to some or all of the separated proteins on the strip will occur. The antibody–antigen complexes are visualized as bands on the strip by using an enzyme-labeled anti-human antibody followed by a chromogenic substrate. Each band corresponds to a unique set of antibodies directed against its respective viral protein. For years, the HIV Western Blot assay has been the gold standard assay for confirmation of HIV antibodies following initial screening with an ELISA (178).

Strip IB assays, also known as line immunoassays, are similar to Western blot except for the preparation of the membrane strip (155). They use the same general procedure for incubation and readout of a chromogenic enzymatic product at the site of binding of a virus-specific antibody. However, strip IB utilizes recombinantly derived proteins and synthetic peptides of a virus that are directly deposited and immobilized at predetermined positions on the membrane. These artificial antigens reduce the presence of contaminating material derived from whole virus lysates and cell culture which may interfere with the assay. Strip IB assays are used mainly as a confirmatory or supplemental test to help verify the specificity of positive results obtained from SPIA used to initially screen for virus-specific antibodies. Commercial kits are available for HSV-1 and HSV-2, HIV-1 and HIV-2, HCV, human T-lymphotropic virus type-I (HTLV-I), and -II (HTLV-II). These assays are less sensitive than EIA screening assays but more specific with fewer indeterminate results compared to Western blot. However, they are relatively expensive and the findings can be subject to interpretation.

Immunochromatographic Assays

IC tests, also known as lateral-flow tests, are a combination of SPIA and immunoblot. In most assays, blood or diluted serum is deposited at the bottom of a membrane. Virus-specific antibodies bind to a solid phase composed of recombinant viral antigens or peptides and a detector reagent. As this solid phase migrates across the membrane by capillary flow, it interacts with antibody capture lines deposited across the membrane. A built-in control line confirms proper functioning of the device after addition of the patient's sample and adequate procedural application. The result is read visually. The advantages of these qualitative assays are that they are simple to use, do not usually require special instrumentation, allow quick turnaround time,

are easy to interpret, and are applicable to urgent, field, and point-of-care testing (179). Commercial devices have been developed in recent years notably for HIV with sensitivities comparable to those of conventional EIA.

IgM Antibody Determination

Virus-specific IgM antibodies are most commonly detected using SPIA and IFA (180). The methods are similar as those used for detecting IgG antibodies, except that IgM bound to viral antigen on the solid phase is detected using secondary anti-human IgM antibodies labeled with suitable markers. Assays to detect virus-specific IgM have been developed for most human viruses, and commercial reagents and complete diagnostic kits are available for a number of the agents (Table 8). However, false-positive and false-negative reactions are major concerns when measuring virus-specific IgM antibodies (180) False-negative reactions are the result of high levels of specific IgG antibodies competitively blocking the binding of IgM to the viral antigen placed on the solid phase. False-positive reactions occur when sera contain unusually high levels of rheumatoid factor (RF) or other interfering substances. RF is an IgM class Ig that reacts with IgG and is produced in some rheumatologic, vasculitic, and viral diseases. In the presence of virus-specific IgG antibodies, RF will form a complex with the IgG molecules. The IgG can then bind to the viral antigen on the solid phase, carrying nonviral IgM antibody with it and resulting in a false-positive result. The incidence of these false-negative and false-positive results can be minimized by separation of IgG and IgM from sera before testing.

A variety of methods have been developed for the removal of interfering RF and IgG molecules from serum, resulting in more reliable IgM tests (181). IgG and IgM antibodies can be physically separated using gel filtration, ion exchange chromatography, affinity chromatography, or sucrose density gradient centrifugation. Although such techniques are effective for separation of IgG and IgM, they are not very practical for clinical use. More rapid and simple procedures have been used for the selective absorption and removal of the IgG fraction from serum using hyperimmune anti-human IgG, staphylococcal protein A, or recombinant protein G from group G streptococci. These pretreatment methods are readily available and are incorporated within most commercial IgM detection kits.

Reverse capture solid-phase IgM or μ-capture assays have been developed as an alternative to the physical fractionation of serum. In this method, the solid phase is coated with an anti-human IgM antibody that is used to capture the virus-specific IgM from the serum specimen. This is followed by washing to remove competing IgG antibody and immune complexes that may interfere with the accuracy of the test. A specific viral antigen is then added and allowed to bind to the captured IgM. The antigen–antibody complexes are detected by adding an enzyme-conjugated secondary antibody, followed by a chromogenic substrate. IgM capture assays are very sensitive and specific and are considered to be superior to other IgM assay formats. An additional background subtraction step can further reduce nonspecific reactivity from interfering substances in serum (182).

TABLE 8 Examples of viruses for which IgM serological determinations are useful and commercial reagents and/or kits are available

Measles virus	Dengue virus
Mumps virus	Hantavirus
Rubella virus	CMV
Parvovirus B19	EBV
Hepatitis A virus	VZV
Hepatitis B virus	HHV-6 and -7
West Nile virus	HSV-1 and -2

Interpretation of Serology Test Results

Interpretation of results for virus-specific antibodies in clinical virology is summarized in Table 9. Demonstration of a seroconversion from a negative to a positive IgG antibody response, or detecting the presence of virus-specific IgM, can be diagnostic of primary viral infection. Differences in antibody titers between acute- and convalescent-phase sera may support a recent viral infection due to reactivation or reinfection, although such testing is retrospective and has a limited impact on patient care. A significant change is defined as a 4-fold or greater rise in IgG titre between acute and convalescent serum when tested in the same assay run. This only applies to methods (e.g., CF, HI, others) that yield results in serial dilution endpoints or international units. Interpretation of a significant difference must rely on objective criteria specified by the laboratory or manufacturer when results of assays such as SPIA are expressed in optical density, relative luminescence, or index values. In titer, 4-fold decreases are seldom observed early enough to be useful for laboratory diagnosis since antibody levels tend to decline slowly over several months after infection. Detection of virus-specific IgG in a single-serum specimen, or no change in antibody levels between acute- and convalescent-phase sera indicates exposure to a virus some time in the past. Negative serum antibody titers may exclude viral infection.

The identification of intrathecal virus-specific antibody production in CSF can confirm the diagnosis of viral CNS infection (146). Finding virus-specific IgM antibodies in CSF is strong evidence for CNS disease caused by arboviruses, LCV, measles, mumps, or rubella viruses. Similar to using serum, 4-fold rises in virus-specific CSF IgG titers can also be diagnostic for these agents, but are less practical because of the need for and delay in testing acute- and convalescent-phase CSF specimens. The detection of any virus-specific antibodies to rabies virus in the CSF is diagnostic of active infection.

When assessing a newborn for congenital viral infections, the presence of IgM in the infant strongly suggests infection since IgM antibodies do not cross the placenta. Seronegative results in both the mother and infant indicate that the suspected viral agent is very unlikely to have infected the infant. Comparison of virus-specific IgG antibody titers between the infant and the mother is essential; a lower titer in the infant most likely reflects passive transfer of maternal antibody, whereas higher antibody titers in the infant than the mother may reflect active antibody production and thus infection of the infant. If maternal and infant serum antibody titers are the same, additional serum from the infant should be obtained 1 to 2 months later and periodically thereafter for 6 to 9 months, to be tested and compared with the earlier antibody titer. These sera should show a decrease in virus-specific antibody relative to the first specimen, if the infant has not been congenitally or perinatally infected with the tested agent.

The results of serologic tests for the detection of virus-specific antibodies must be interpreted with caution, because measurements of an antibody response to viral infections can be complicated by a number of factors. There may be a lack

TABLE 9 Interpretation of results for virus-specific antibodies in clinical virology

Context	Result	Interpretation
Immune status	IgG present	Past exposure or immunization
	IgG absent	Past exposure unlikely
Diagnosis of acute infection	IgM present	Primary or recent viral infection likely
	IgG seroconversion	
	Fourfold rise in IgG titers	
	Absence of IgM or IgG	Excludes viral infection
	IgG present, single or stable titer	Past exposure
CSF infection	IgM present	Strong evidence of CNS infection
	IgG seroconversion	
Congenital infection	IgM in infant	Evidence of congenital infection
	Absence of IgM or IgG in mother and infant	Suspected infection unlikely
	IgG infant < IgG mother	Passive antibody transfer
	IgG infant = IgG mother	Retesting in 1 to 2 months required
	IgG infant > IgG mother	Suggests congenitally or perinatally acquired infection
Immunocompromised individual	IgM and/or IgG absent	Possible delay in production of serum antibodies

of or delay in production of serum IgM or IgG antibodies, particularly in newborns, the elderly, and immunocompromised hosts. IgM antibodies also may persist for extended periods after primary infection and can be present during reactivation of latent viral infections. IgM may be undetectable during acute disease for individuals that were previously immunized but unsuccessfully protected. Significant rises in IgG antibodies do not always occur as a result of recurrent infections or exogenous reinfection. Virus-specific IgG antibodies may be present in recipients of intravenous Ig, newborn infants possessing passively acquired maternal antibody, or patients who have received recent blood transfusions, making it difficult to interpret IgG tests. Rises in the titres of either IgM or IgG antibody to certain viruses also may be nonspecific and in response to recent infections with other viruses. Accordingly, the possibility of a false-positive IgM result should be considered when the incidence of the virus under investigation is low. Indeterminate results inherent to some immunoassays may require additional patient visits, venipuncture, and repeated serologic testing. Accuracy of antibody detection assays is critical when follow-up care decisions are based on test results. For this reason, serologic assays producing fewer inaccurate or indeterminate results than others are likely to be more cost-effective. Finally, failure to establish an accurate serological diagnosis frequently results from the inability to submit an adequate set of paired serum.

For neurological viral diseases, the appearance of virus-specific antibody in the CSF may be delayed for 2 to 4 weeks. Also, for certain viruses, the presence of virus-specific antibody in the CSF may represent the passive transfer of serum antibodies across a damaged blood–brain barrier. Methods must be performed to determine and compare the CSF/serum ratio of virus-specific antibody to the CSF/serum ratio of albumin, particularly for members of the *Herpesviridae* family (146). Since albumin is not synthesized in the CNS, its presence in high concentrations within the CSF reflects the presence of contaminating serum proteins and an interruption of the blood–brain barrier. Demonstration of an intact blood–brain barrier in the presence of high levels of detectable virus-specific CSF antibody represents intrathecal production of antibody and is considered evidence of viral infection of the CNS.

In the diagnosis of an infant with suspected viral congenital disease, measuring IgG antibody titres to the common agents rarely results in a definitive diagnosis and is more useful for excluding infection. Detecting a single elevated titer of IgG antibody to a specific viral agent is not useful and testing for HSV-specific antibody is of limited value. Negative antibody titers in the mother and child also may be the result of the mother having a primary infection of recent onset without immediate production of virus-specific antibodies. Appropriate follow-up studies are essential. Because of the many caveats associated with serologic diagnoses of viral infections, antibody determinations should be confirmed by isolation of the virus in culture or by using direct methods of detecting viral antigens or nucleic acids. The results should also be interpreted with careful consideration of the patient's symptoms and history.

QUALITY ASSURANCE AND QUALITY CONTROL

Quality management, assurance, control, and continuous improvement are key elements of a complete quality assessment system, ensuring that all steps from specimen collection to result reporting are performed correctly. The goal of maintaining a quality system in a diagnostic laboratory is that "the right test is performed on the right specimen, and that the right result and interpretation are delivered timely to the right individual."

Quality Assurance

Quality assurance is a broad term used to describe administrative and technical procedures for monitoring quality in the laboratory. Licensed, certified, or accredited clinical virology laboratories must comply with specific requirements of comprehensive quality assurance programs that are issued by regulatory and accreditation agencies. In the U.S., some minimal requirements are supported by legislative measures. In other countries, their scope may vary depending on local or regional jurisdictions. Quality assurance requires active

participation by all members of the institution. Standard operating procedures specify how preanalytical, analytical, and postanalytical activities are to be performed. These procedures are revised periodically and updated when necessary. Internal audits performed at regular intervals monitor compliance. Record keeping, equipment calibration and preventive maintenance, personnel training and competence assessment, and internal and external proficiency testing are all part of a quality assurance program (183). Ultimately, quality assurance leads to improvement of all aspects of laboratory services so that patient healthcare will benefit.

Quality Control

Quality control focuses on monitoring the testing process itself in order to ensure test system performance. Variables affecting the quality of results are adequacy of training of laboratory personnel, sample type and condition, assay reagents, equipment performance, testing procedure deviance, and interpretation, transcription, and reporting of test results. Key elements of quality control include a set of written procedures specifying the elements to verify prior, during, and after the testing process. Major issues to be considered in this regard are documenting instrument conditions (e.g., temperature); calibration and use; reagent labeling regarding receipt, storage, and use prior to expiration date; storage of materials; hazard materials labeling and use; comparison of new methods to prior standards; and satisfactory performance of kits. Initial qualification of new lots of kits and reagents using known standard panels and insertion of control samples on each test run ensure that the kit, device, or equipment is performing within specifications and that the results generated are correct. For in-house assays, performance characteristics must be established. These include repeatability and reproducibility, cutoffs for qualitative assays, and linearity including lower and upper limits of quantification for quantitative and semiquantitative assays.

Monitoring test performance may allow detection of factors affecting assay quality, and ultimately reliability of results. Analyzing data for trends can establish deviation in test results over time (e.g., drift in the performance of a manufactured diagnostic kit). Investigation of a faulty test or run is facilitated when ancillary information is documented, and thus traceable throughout the procedure. If a result is not obtained according to procedure, or a specific instrument or reagent has failed to meet prescribed criteria, a nonconformity to a test procedure should be documented as well as the corrective action that is put in place to remedy the problem. This compilation is essential as it can identify a weakness in assay performance that otherwise may go unnoticed.

Analyzers and robotic platforms interfaced with computers are more common now in the virology laboratory, especially for antigen, antibody, and nucleic acid detection assays. Bar coded clinical specimens and reagents limit errors and facilitate monitoring and documentation of tests parameters. Users have the responsibility to assess the performance of these automated instruments. As test results are required rapidly to ensure prompt medical decisions, the diagnostics industry has been responding with point-of-care devices or tests, such as for influenza, RSV, and HIV. These assays are aimed to be used outside of the strictly controlled laboratory realm and constitute a new challenge for quality control in clinical virology. Development of adequate built-in controls may help to ensure accuracy of the kit result. However, deficiencies regarding basic documentation, training, conformity of testing to manufacturer's package insert, respect of expiration dates, and knowledge of the limits of the test may affect result quality and interpretation. The consequences of an inaccurate result may include inappropriate medical decisions, unnecessary interventions, and worry for the patient. Therefore, personnel performing point-of-care tests should comply with documented protocols for specimen collection, processing, and testing, including adherence to established guidelines for confirmatory testing. It may also be considered to retest negative samples with an alternative assay when point-of-care testing is used to support prophylactic decisions (e.g., HIV). Finally, users should initiate corrective action when deviations or nonconformities to established parameters are detected.

Practical aspects of quality assurance programs and quality control in the clinical laboratory can be found in several American Society for Microbiology documents including Clinical Microbiology Procedures Handbook and Cumitechs (http://www.asm.org/). The Clinical and Laboratory Standards Institute has also published guideline documents addressing quality assurance and quality control issues for viral culture, immunoassays, point-of-care testing, and molecular diagnostic methods (http://www.clsi.org/).

This chapter is dedicated to the memory of Michel Couillard, our co-author in the third edition.

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Immune Responses to Viral Infection

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The concept of "immunity" dates back to ancient Greece, where as early as the fifth century BC, documented cases of "immune" individuals were described who were related to individuals who recovered from the plague (1). However, it was not until the 10th century that specific "interventions" were described that could induce immunity. In both China and the Middle East, a process known as "variolation," consisting of purposefully exposing healthy individuals to the contents of dried variola lesions, was actively practiced to prevent severe infection with smallpox. In the early 18th century, the practice of variolation was brought to Great Britain, where the development of the first vaccine by Jenner catalyzed the creation of the field of immunology. Beginning in the late 19th century, major breakthroughs, including the establishment of the "germ theory" by Koch and Pasteur, which held that disease was caused by bacteria or pathogens; the discovery of phagocytic cells by Metchnikoff; the identification of immune proteins in serum by von Behring and Kitasato; the identification of B cells and their regulation by Ehrlich; the discovery of lymphocytes by Gowan; the identification of pattern recognition receptors and innate immune activity by Janeway; and the discovery of dendritic cells, which link the innate and adaptive immune system, by Steinman, collectively gave rise to modern immunology and our current appreciation for the host immune response. This response has evolved to contain, eliminate, and remember virtually any pathogen to which it is exposed. This chapter reviews the components of the immune system, focusing on the innate and adaptive immune response to viral infection and how these arms of the immune system collaborate to prevent and control viral disease.

THE IMMUNE SYSTEM

The immune system consists of a network of highly specialized cells, tissues, and organs that work collectively to provide protection from infection. Immune protection can be broadly divided into three lines of defense: (1) **barrier immunity** that nonspecifically protects against the invasion of pathogens, (2) **innate immunity** that is comprised of specialized networks of cells that rapidly respond, in a non-antigen-specific-manner, to control and clear the invading pathogen, and (3) **adaptive immunity** that is a slower cellular response that evolves during an infection to confer antigen-specific memory against that pathogen for life. Importantly, these three lines of defense, discussed below, collaborate to effectively prevent, contain, and eliminate infection via cellular communication networks, where at each line of defense, a failure to contain the threat is communicated to the next line of defense to ensure that immunity develops rapidly to contain the invading pathogen. The importance of these immune networks is most evident in genetic defects that result in compromised immunity that are often associated with severe disease (Table 1). For example, more than 200 inherited genetic defects have been described that affect single points within the immune response, and only very few children can survive such abnormalities.

Barrier Immunity

The human body contains over 100 trillion cells. However, only 1 in 10 of these cells is human; the rest of the cells are largely bacterial and fungal. While the vast majority of this microbiome is innocuous or even beneficial to the host, some of these microorganisms would be pathogenic if allowed to proliferate unfettered. Recently, a virome, consisting of the collection of uncontrolled/infectious, persistent/chronic, genomically integrated, and asymptomatic viruses, has been described that may play an equally important role in both shaping the bacterial microbiome (2) and modulating and supporting the immune system (3). Thus, the first line of defense includes physical and chemical barriers that cover the surfaces of the body exposed to the environment (Fig. 1). These barriers, consisting of specialized cells and molecules, create a physical barricade that viruses must traverse and include (1) the skin, (2) mucosal membranes that line organs exposed to the external environment, and (3) the microbiota present on these barrier surfaces. Together, these barriers create highly complex physical and chemical barricades to prevent infection with incoming pathogens.

The Skin

The skin is the body's largest organ, constituting a major component of our first line of defense against infection. Although there are many potential pathogens on the surface of the skin, the skin regularly repels these invaders and is rarely infected by direct inoculation of viruses with the exception of some poxviruses, human papilloma virus (HPV),

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TABLE 1 Clinical manifestations of genetic defects in different arms of the immune system

Immunological pathway	Defect	Immunodeficiency or syndrome	Mutated gene	Key clinical manifestations
Defects in PRR	Lack of TLR3, TLR7, TLR8, TL9 signaling	UNC93B deficiency	*UNC93B1*/UNC93B1 protein	Impaired cellular responses to IFN-α/β and -λ, increased infections with **HSV** and **CMV**
	Lack of TLR3, signaling	TLR3 deficiency	*TLR3*/TLR3	Impaired cellular responses to IFN-α/β and -λ, increased infections with **HSV** and **CMV**
	Impaired signaling from TLRs except TLR3	MyD88 deficiency, IRAK4 deficiency	*MYD88, IRAK4*	Invasive and severe infection with S. pneumoniae, S. aureus, and P. aeruginosa, but normal response to other bacteria, viruses or parasits
	Impaired TLR3 signaling	TRAF3 adaptor molecule deficiency	*TRAF3*/TRAF 3	Impaired TLR3-dependent induction of type I IFN and IFN-λ, increased **HSV** infections, otherwise healthy
	Lack of NFκB activity resulting in lack of TLR and other PRR signaling	NEMO defects	*NEMO*	Resulting in lack of intracellular signaling including TLR signaling, lethal
Defects in phagocytes/ granulocytes	Neutropenia	WHIM syndrome	*CXCR4*/chemokine receptor 4	Retention of neutrophils in the bone marrow, decreased IgGs, **HPV** infection
	Extreme neutrophilia	Leucocyte adhesion defect/ LAD	Integrin beta2	Skin infections and poor wound healing
	Absence of specific granules	Neutrophil-specific granule deficiency	CCAAT	Increased pyogenic infections
Defects in NK and T cells	Lack of SLAM-associated signaling (SAP)	XLP syndrome 1	*SH2D1A*	T and NK cell lymphoproliferative disorder, dysgammaglobulinemia; severe **EBV** infection
	Lack of SLAM-associated signaling (SAP)	XLP syndrome 2	*BIRC4*/ X-linked inhibitor of apoptosis	Reduced survival of NK cells, NK lymphopenia, increased apoptosis of T cells, hypogammaglobulinemia, severe **EBV** infection
	T-cell signaling transfuction	EBV-associated lymphoproliferative syndrome	*Itk*/interleukin-2 inducible T-cell kinase	Absent NK cells, severe **EBV** infection
	Defect in T-cell activity	Chronic active EBV infection	*PRF1*/perforin 1	Decreased cytotoxic T cell and NK cell activity, hypogammaglobulinemia, severe **EBV** infection
	Unknown/ zinc homeostatis	Epidermodysplasia verruciformis	*EVER1*,*EVER2*/epidermodysplasia verruciformis 1 and 2	Decreased T-cell responsiveness, fulminant **HPV** infection
	Overactive T cells with lack of T regulatory control	X-linked immune dysregulation with polyendocrinopathy (IPEX) Syndrome	FoxP3	Autoimmune diseases
Defects in humoral immunity	Impaired CD4 B-cell interaction and AICDA activation	Hyper-IgM syndrome	Type 1: CD40L, Type 2: AICDA, Type 3: CD40, Type 4: downstream AICDA, Type 5: UNG	Hypergammaglobulinemia, lack of class switching, recurrent viral, bacterial, and parasitic infections
	Defect in B-cell maturation	X-linked agammaglobulinemia	*BTK*/Bruton's tyrosine kinase	Hypogammaglobulinemia/Agammaglobulinemia, low B cell numbers, **Enterovirus** infections

NEMO = Nuclear factor-kappa B essential modulator; WHIM = Warts, Hypogammaglobulinemia, Immunodeficiency, and Myelokathexis; SLAM = signaling lymphocytic activation molecule; XLP = x-linked lymphoproliferative; AICDA = Activation-induced cytidine deaminase.

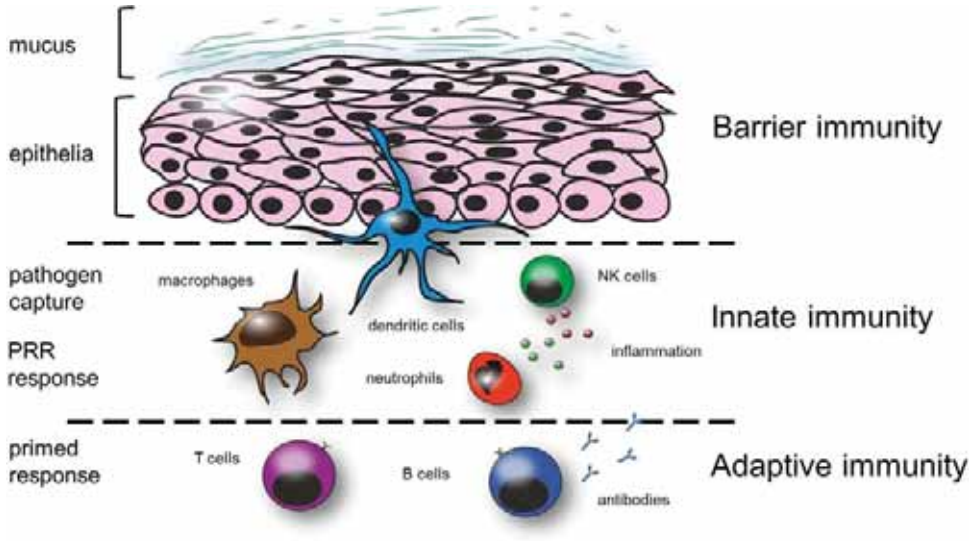

FIGURE 1 Three layers of immunity: physical barrier immunity, innate immunity, and adaptive immunity. 1) Physical barriers, including the skin and mucosal membranes, represent the first level of protection against foreign invaders. Damage to this layer can lead to pathogen access to the underlying cells. Many viruses utilize vectors to breach this barrier. 2) Innate immunity represents the nonspecific rapid response arm of the immune system, and the first white blood cell responders help control, clear, and arm the immune response. C) Adaptive immunity consists of the antigen-specific response that evolves to specifically clear the pathogen and to confer long-lived immunity against the pathogen upon re-exposure.

herpes simplex virus (HSV), or polyoma viruses. While the skin functions largely as a physical barrier, it also dynamically participates in immune surveillance as it actively secretes a number of compounds with antimicrobial and inflammatory activity and is home to a multitude of effector cells of both the innate and adaptive immune systems.

The primary function of the skin is to protect the body from a range of environmental threats, including temperature, radiation, chemicals, and microorganisms (Fig. 1). It is composed of two main layers, the epidermis and the dermis, with the outer epidermis functioning as a passive barrier to infection. The epidermis is largely comprised of terminally differentiated keratinocytes, and by itself, the keratinized epidermis is an impenetrable barrier to most viruses. It is only upon injury to the skin that pathogens are able to penetrate and infect the underlying tissues, such as in the case of burns, which render this barrier remarkably vulnerable to pathogens. Thus, some viruses, such as Japanese encephalitis virus, dengue virus, and yellow fever virus, have evolved mechanisms to breach this barrier by entering the host via arthropods (e.g., mosquitoes) that deposit the pathogens directly into the underlying dermis.

The skin itself can also actively limit a potential infection via the production of a number of compounds with antimicrobial and inflammatory activity produced by a variety of skin-resident cells, including keratinocytes, sebocytes, eccrine glands, and mast cells, as well as cells that are recruited to the site of infection, such as neutrophils and natural killer cells (4). Although viral skin infections are a less common manifestation of primary immune deficiencies (PIDs), compared to bacterial infections, severe warts due to human papilloma virus (HPV) do emerge in some individuals with immune PIDs. For example, epidermodysplasia verruciformis (EV) is associated with uncontrolled HPV wart proliferation and eventually malignant transformation due to defects in genes involved in zinc metabolism (5). Additional gene defects in GATA-2 and serine threonine kinase 4 (STK4) mutations have also been linked to disseminated facial warts due to HPV. Moreover, allergy-associated eczema and autoimmune psoriasis, both of which alter the integrity of the skin barrier, have been indirectly associated with a predisposition to viral infection.

Although the skin is home to a large number of distinct immune effector cells discussed in more detail below, including macrophages, natural killer cells, and mast cells, one of the most important skin-resident immune cells is the Langerhans cell (6). Langerhans cells are a class of antigen-presenting cells, or dendritic cells, that act as critical gatekeepers of the immune system and are responsible for capturing pathogens within skin, processing foreign antigens, and traveling to specialized immune organs (lymph nodes) to present these antigens and instruct cells of the adaptive immune system. The adaptive immune cells then travel back to the site of infection to contain and clear the infection. Ultimately, both skin-resident and recruited immune cells, as well as the local skin cells, collaborate to clear the pathogen and ensure that the integrity of the tissue is maintained.

Mucosal Barriers

Other tissues of the body are also directly accessible to the environment, including the gastrointestinal, respiratory, and urogenital tracts (7). Whereas the skin is covered with a thick keratinized layer, these other tissues typically require moisture to mediate their biological activities and thus are not coated with a water-impermeable keratin, like the skin is. Rather, these internal tissues are protected by a mucosal barrier, that is, thinner layers of epithelial tissue that are coated in a thick fluid known as mucus (8). However, because many microorganisms grow prodigiously on moist barriers, these surfaces are littered with symbionts and potential pathogens and are therefore often more susceptible to infection. Similar to the skin, mucosal surfaces possess an array of strategies aimed at protecting these vulnerable epithelial barriers, including chemical barriers, the production

of immunomodulatory proteins, and a large population of tissue-resident immune cells that are armed and ready to respond upon infection.

A thick layer of keratin protects the skin, and a thick layer of mucus protects mucosal membranes. Mucus is a viscous, acidic fluid that functions to both lubricate the tissue and protect the tissue from potential pathogens. It is produced from specialized cells found within the mucosal barrier (e.g., goblet cells in the intestinal mucosae) and largely consists of heavily glycosylated proteins (e.g., mucins), other glycoconjugates, and water. These components form interconnecting lattices within the mucus that protect the underlying epithelium via the formation of a thick (over 100 μm), dense network that pathogens must traverse to access the underlying epithelial border. Although small molecules can easily diffuse through the mucus, movement through this fluid is remarkably difficult for pathogens, including viruses that are trapped easily (9), as is evidenced by the lack of any microorganisms in the mucus adjacent to the epithelial barrier. Intriguingly, the epithelial-proximal mucus glycoproteins are often transmembrane proteins with large cytoplasmic tails containing multiple signaling motifs (10). Therefore, it is possible that some mucus proteins may function in two capacities, in limiting pathogen access to the underlying barrier and also as sensors that may signal into the underlying epithelium should a potential pathogen make it through the mucous layer. This may enable the epithelial border to secrete antimicrobial and inflammatory proteins and alert underlying immune cells to restrict any pathogens.

As injury to the mucosal epithelium can have grave infectious consequences, similar to injury to the skin, the mucosal epithelium secretes a large number of antimicrobial compounds that can directly kill invading pathogens or recruit and activate tissue-resident and circulating immune effector cells. These antimicrobial compounds are secreted by specialized cells in the epithelium (e.g., Paneth cells in the intestine) and can diffuse through the mucus, providing an additional layer of protection from infection. Furthermore, mucosal epithelia are lined with large numbers of both innate and adaptive immune cells, including T and B cells, dendritic cells, neutrophils, natural killer cells, and macrophages. These tissue-resident immune cells patrol for invading pathogens and evidence of stress due to an epithelial breach and form lymphoid aggregates that enable the rapid induction of mucosal-specific immune responses. Thus, even though mucosal surfaces are under constant exposure to external threats from the air, food, and commensal microbiota, a combination of structural, chemical, and cellular components protects these vulnerable sites from infection.

Commensal Organisms

Although the body's barrier surfaces are designed to prevent infection with and control the commensal microbiota that colonize these surfaces, it has become increasingly apparent over the past decade that the commensal microbiota also provide protection against potentially pathogenic microorganisms (11, 12). Because the commensal microbiota significantly outnumber the cells in our body, these microorganisms also play a prominent role in preventing infection both directly via production of natural antimicrobicides and indirectly via cross-talk with the underlying epithelium and immune effector cells that may render the barrier more resistant to viral infection (13). The critical nature of the commensal microbiome in providing protection from infection has been most clearly demonstrated in the setting of long-term antibiotic treatment, where disruption of the natural microbiome results in the overgrowth of pathogenic yeast and bacteria such as *Candida* spp. and *Clostridium difficile*. Moreover, it has also been demonstrated in some viral infections, such as influenza and HIV infection, that particular microbial flora are critical for the evolution of effective antiviral immunity (14, 15) and even in providing a more fertile ground for HIV transmission at the mucosal barrier (16). Furthermore, the use of probiotics or stool transplantation has been shown to reverse disease associated with pathogen colonization, clearly illustrating the critical nature of the microbiome itself as a barrier to infection.

Moreover, beyond the microbiome, an enormous and highly diverse population of viruses that shape immunity coexist with humans (17). This other "microbiome" includes viruses that cause symptomatic and nonsymptomatic infections that may infect the host and/or the microbiome, that may coexist or integrate into the host genome, and that can even be reactivated intermittently and cause *de novo* infections. Moreover, recently it was shown that the HIV infection-induced changes in the enteric virome, specifically related to the expansion of enteric adenoviruses, were associated with a loss of CD4+ T-cell counts and therefore disease progression (18). Moreover, this altered virome was highly associated with dramatic alterations in microbial biodiversity, pointing for the first time to a role for the enteric virome in driving AIDS-associated enteropathy and disease progression.

Innate Immunity

Intertwined with physical barrier immunity, the innate immune system provides an immediate response to infection and foreign antigens (Fig. 1). The innate immune system is a network of cells and molecules that is rapidly mobilized in response to danger signals generated following infection. Importantly, the innate immune system, composed of a network of distinct effector cells (Fig. 2), is armed and ready to respond without the need for prior antigen sensitization. Because the innate immune system is able to fight pathogens at the site of infection, it provides an immediate defense against any incoming pathogens.

Effectors of Innate Immunity

The innate immune system is composed of five major classes of immune effectors that collectively aim both to contain the infection as well as to provide the infrastructure necessary for the generation of a long-lived adaptive immune response that can drive long-term memory against that pathogen: (1) phagocytes, (2) granulocytes, (3) innate lymphoid cells, (4) antigen-presenting cells, and (5) the complement system. These innate immune cell types and proteins, each with a highly specialized function, have evolved to rapidly recognize, contain, and eliminate invading pathogens.

Phagocytes

Tissue-resident phagocytes are typically the first line of defense against pathogens that have broken through protective barriers. Phagocytes are cells that protect the body through a process by which a cell internalizes a smaller cell, cell fragment, microorganism, or foreign particle (called phagocytosis). While many cell types possess phagocytic activity, the principal phagocytes in the body are macrophages, neutrophils, and dendritic cells (discussed further below).

Macrophages are mononuclear phagocytes that mature from blood monocytes that have left the circulation and migrated into the tissue (19). They are relatively long-lived,

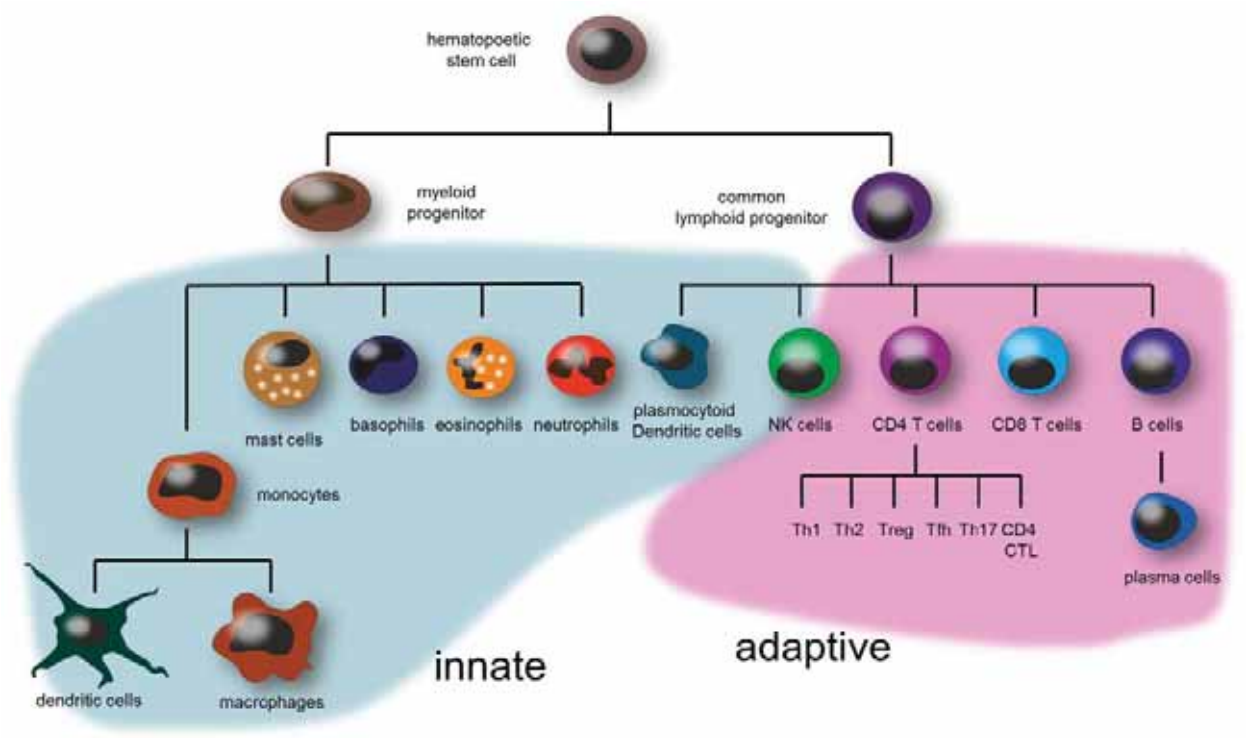

FIGURE 2 The two arms of the immune system. All immune cells develop from a common hematopoietic stem-cell progenitor that can differentiate into a myeloid or common lymphoid progenitor (CLP). The adaptive arm of the immune system is composed of four immune cell types, called lymphocytes, that share a CLP origin. The innate arm consists of an array of distinct cell types, most of which are derived from the myeloid progenitor, except NK cells and plasmacytoid dendritic cells that differentiate from a CLP.

with a lifespan of several months. Unlike other phagocytes, which are typically recruited to the site of infection, macrophages can be found in healthy, uninflamed tissue, making macrophages critical sentinels against infection and the first innate immune cells to respond to infections. Macrophages and other phagocytes recognize pathogens via the use of cell surface receptors (detailed below) that can discriminate pathogens from self. Upon recognition of the pathogen via these receptors the pathogen is actively engulfed and killed by the phagocyte.

In addition to their phagocytic activities, macrophages also play a critical role in inducing inflammation to recruit additional immune effector cells to the site of infection. Macrophages are constantly surveying their surroundings for pathogens. Upon infection of the host, the activation of macrophages leads to the rapid release of copious amounts of proinflammatory cytokines and chemokines that arm the immune system, recruit additional cells, and drive enhanced pathogen clearance. This inflammatory cascade triggers the rapid recruitment of additional waves of macrophages and neutrophils to the site of infection. The newly recruited macrophages are vital to the rapid clearance of dead and dying neutrophils, as well as to the clearance of pathogens or pathogen-infected cells, which can be rapidly processed and presented in lymph nodes for the induction of adaptive immune responses to promote long-lived adaptive immune memory.

Given their critical role in the initiation of the immune response, several viruses deliberately infect macrophages, including adenoviruses, alphaviruses, HIV, ebola virus, and dengue virus (20), and impair the immunological activity of these cells from within. In the context of HIV infection, the virally encoded proteins directly interfere with the antigen-presenting activities of macrophages via the downregulation of major histocompatibility complex antigens (MHC) II and CD4. Despite this HIV-evasive activity, there is ongoing debate whether infection of macrophages represents a critical safety niche for HIV, because once infected, these macrophages can take up long-lived residence in tissues throughout the body, including immune-privileged sites such as the brain (21).

While macrophages are typically the first phagocyte to respond to an infection, **neutrophils** are the most abundant phagocyte in the body and first to invade sites of infection (22). Neutrophils are short-lived (a lifespan of approximately 5 days), polymorphonuclear cells that make up the majority of the white blood cells in the body, and their main function is to phagocytize and destroy invading pathogens. Unlike macrophages, however, neutrophils are not typically found in healthy, uninflamed tissue; rather, they are rapidly recruited to sites of infection en masse by chemokines secreted by macrophages and other cell types in response to infection. Upon recruitment, they are involved in the rapid phagocytic clearance of invading pathogens.

In addition to their role in the phagocytic elimination of pathogens, neutrophils are also armed with a number of cytoplasmic granules containing enzymes and antimicrobial peptides. Two different types of cytoplasmic granules can be found in neutrophils, depending on their maturation state. Immature neutrophils possess primary granules loaded with cationic proteins and enzymes involved in protein degradation (e.g., elastase and cathepsins) and pathogen destruction

(e.g., defensins, lysozyme, and myeloperoxidase). By contrast, more mature neutrophils are loaded with secondary granules that contain enzymes that are involved in generating reactive oxygen species. When stimulated by a pathogen, the contents of these granules are rapidly released into the extracellular environment, where they indiscriminately kill surrounding cells, a process known as degranulation. Because the uncontrolled degranulation of neutrophils can lead to extensive tissue damage, this process is tightly regulated.

While the ability of neutrophils to eliminate pathogens via phagocytosis and the degranulation-mediated release of antimicrobials has long been known, the third strategy employed by neutrophils to eliminate pathogens has only recently been discovered. Activated neutrophils can release a web-like DNA structure known as a neutrophil extracellular trap (NET). These traps are comprised of a web of chromatin and serine proteases that can snare and kill extracellular pathogens in the absence of phagocytosis. Furthermore, these traps can function as a physical barrier to limit the progression of the infection.

While neutrophils are critical for immune defense against bacteria and parasites, their role in antiviral immunity has only begun to be elucidated (23, 24). Multiple viruses, including influenza virus, herpes simplex virus, respiratory syncytial virus, and cytomegalovirus, have been shown to activate neutrophil phagocytosis, degranulation, and NET release. Furthermore, the depletion of neutrophils results in a compromised immunity against influenza virus (25). NET formation and release is critical for the early control and clearance of poxvirus infection in the liver, illustrating the role of neutrophils in distinct tissues and against diverse viruses. Importantly, neutrophils are not only involved in the direct antiviral response to infection but also critically contribute to shaping the adaptive immune response via their unique localization and activity in the lymph node where they are involved both in limiting antigen availability (26) and driving enhanced T-cell proliferation (27).

Granulocytes

In addition to being a phagocyte, neutrophils are also a member of a larger family of cells known as granulocytes. Granulocytes are white blood cells that are characterized by the presence of numerous granules in their cytoplasm. These granules contain a number of antimicrobial and immunomodulatory compounds, and while granulocytes can have secondary functions in antigen presentation, the principle function of granulocytes is the release of these compounds into the extracellular space where they can directly kill potential pathogens, induce inflammatory cascades, and recruit other immune effectors. There are two additional types of granulocytes, eosinophils and basophils, and while not technically granulocytes, mast cells are very similar to basophils and are discussed here.

Eosinophils are highly granular, myeloid-derived white blood cells that are largely involved in the antiparasite immune response. Following activation, eosinophils are stimulated to release a number of cytotoxic cationic granule proteins, including proteins with RNAse activity (28), antimicrobial reactive oxygen species, proinflammatory cytokines, and potent inflammatory mediators known as leukotrienes (29). While eosinophils are not classically considered critical antiviral mediators, increased eosinophil numbers have been implicated in enhanced clearance of respiratory syncytial virus (RSV) infection (30) accumulating in the lungs of RSV infected patients (31), where eosinophils appear to respond directly to the viral RNA. However, the role of eosinophils in anti-RSV protection is not direct; instead, eosinophils dampen inflammation and reduce infection-associated immunopathology in the lung tissues following viral infection (32). Thus, while eosinophils likely contribute to antiviral immunity, their effects are likely largely mediated through their ability to protect the tissues where infections occur.

Basophils are the least frequent myeloid-derived granulocyte in the blood, and like eosinophils, when activated, basophils release a large number of granule proteins and enzymes (e.g., histamine and elastase) and inflammatory mediators (e.g., cytokines and leukotrienes) that promote vasodilation and inflammation. Although a direct role for basophils in the elimination of viral infections has not been described, basophils have several secondary roles that are important in the resolution of viral infections. First, basophils produce large amounts of the cytokine IL-4 in response to viral infection (33). IL-4 is a cytokine important for the development and activation of particular subsets of T cells, humoral immune responses, and innate lymphoid cells (34). Additionally, basophils can tune dendritic cell activity to promote specific adaptive immune responses (35) and have been implicated as the earliest recruiters of eosinophils into inflamed skin (36).

While not technically granulocytes, **mast cells** are large granular innate immune cells that are very similar to basophils (37). Mast cells leave the bone marrow in an immature form, and they remain immature until they enter the tissue, where they mature into highly granular cells, loaded with preformed stores of histamines and various enzymes that are critically involved in triggering inflammatory responses. Thus, within seconds of an infection, mast cells are able to release immunomodulatory compounds that rapidly initiate an inflammatory cascade to selectively kick start the antiviral immune response. In particular, mast cells contain large intracellular stores of histamine (2 to 5 pg/cell) that are rapidly released in response to activation. Histamine is a potent vasodilator that also increases vascular permeability, enabling the recruitment of large numbers of innate immune cells, such as neutrophils and macrophages, to enter infected tissues. In addition to their role in initiating inflammatory cascades, similar to neutrophils, mast cells are able to directly phagocytize pathogens or infected cells, release reactive oxygen species that can directly kill pathogens, and release extracellular traps (38). Thus, while mast cells are most classically known for their role in allergy, they have been implicated in respiratory virus triggered-asthma and are clearly critical for the early defense and arming of the innate immune response aimed at containing viral infections.

Innate Lymphoid Cells

Unlike granulocytes, which are derived from a common myeloid precursor, innate lymphoid cells develop from a common lymphoid precursor cell that also gives rise to the cells of adaptive immune system (B cells and T cells) (Fig. 2). Innate lymphoid cells rapidly secrete immunoregulatory cytokines upon activation and have been broadly divided into three groups based on the cytokines they secrete. Group 1 innate lymphoid cells typically produce type 1 cytokines (e.g., IFN-γ and TNF-α) and include cytotoxic natural killer cells; Group 2 innate lymphoid cells typically produce type 2 cytokines (e.g., IL-4, IL-5, and IL-13); and Group 3 innate lymphoid cells produce IL-17A and/or IL-22. With the exception of natural killer cells, the role of the various subsets of innate lymphoid cells in infection has only recently begun to be elucidated (39); however, given their localization

within mucosal tissues and their ability to rapidly secrete a wide variety of immunoregulatory cytokines, they likely play an important role in the early immune response to infection.

Natural Killer Cells. Natural killer (NK) cells are the only innate immune lymphocytes that survey the periphery for stressed, malignant, or infected cells. These cells develop in the bone marrow, where they undergo a complex educational process on cells expressing self-antigens that select for NK cells that recognize self (major histocompatibility complex, MHC) through inhibitory NK cell receptors, and therefore can be turned off (40). Thus only NK cells that will not become auto-reactive are permitted to mature and gain cytolytic functions, in a process called "licensing" or "education." Importantly, the critical nature of NK cells in antiviral immunity is most clearly illustrated in the case of children born with NK cell deficiencies who are highly susceptible to HSV infection (Table 1), despite the presence of normal adaptive immune responses (41). Moreover, NK cells have been centrally linked to spontaneous control of infection with hepatitis C virus (42) and control of HIV (43), highlighting the essential role of this innate immune cellular subset in antiviral immunity.

Importantly, NK cells are preloaded with cytolytic granules, and upon recognition of a stressed or infected cell, release these granules in a highly regulated and directed fashion. These granules contain perforins and granzymes that generate holes in the membrane of target cells, resulting in the lysis of or the induction of apoptosis of the target cell. Importantly, NK cells are capable of killing any aberrant cell without the need for antigen sensitization.

Because of their highly cytolytic nature, NK cell function is regulated through a complex network of inhibitory and activating receptors. Among the large array of receptors that can be expressed on NK cells, four major receptor families regulate NK cell function. The first family includes the killer immunoglobulin receptors (KIRs) that interact with MHC, expressed on all cells. The MHC proteins are involved in presenting random samples of small segments of self-proteins to the immune system to ensure that the cell is healthy. However, upon infection, viral proteins are rapidly synthesized, offering a novel array of peptides for presentation in MHC to circulating NK cells. Many viral infections, such as HIV (44) and CMV (45), downmodulate the expression of MHC class I, to reduce viral-peptide exposure. However, loss of MHC is sensed by circulating NK cells, signalling that the cell is stressed or infected, and activating NK cells via their KIR receptors. Moreover, emerging data also point to a role of viral peptides that alter the conformation of MHC as critical ligands for KIR. Thus the KIR family arms NK cell activation and cytolytic activity both through a loss of MHC sensing as well as through altered viral peptide conformational changes to MHC (46). The second class of receptors include the C-type lectin receptors (CLRs, described below) that are involved in sensing stress following the up-regulation of a class of MHC homologues (MICA/B or ULPB) that are rapidly expressed following genotoxic or viral infection related intracellular stress (47).

The third class of receptors includes the natural cytotoxicity receptors (NCRs) involved in the rapid activation of NK cells (e.g., by influenza) (48). Self-ligands have yet to be defined. The final class of receptors includes FcγRIIIA (CD16), which is involved in driving antibody-dependent cellular cytotoxicity following the opsonization of virally infected cells with antibodies. Thus, while additional activating and inhibitory receptors are expressed on NK cells, together this large array of germline-encoded receptors that are expressed stochastically on the surface of NK cells resulting in a highly heterogeneous network of NK cells (49) collectively regulate and rapidly deploy the antiviral activity of these cells following viral infection.

While NK cells play a critical role in the nonspecific response to infection, more recent data suggest that a novel subset of NK cells, termed memory NK cells, may also emerge following viral infections (e.g., lymphocytic choriomeningitis infection in mice) and result in long-lived innate memory (50). Moreover, these memory NK cells appear to also evolve following vaccination with distinct viral antigens (51) and can survive up to 5 years in non-human primates following vaccination with particular viral vectors (52). Thus, while the bulk of the NK cell response is involved in a rapid nonspecific cytolytic control of viral infection, a small subset of these innate lymphocytes may emerge following infection that are aimed at conferring long-lived memory and rapid responsiveness upon pathogen re-encounter, suggestive of adaptive-like activity within this unique innate lymphocyte cell subset.

Antigen-Presenting Cells. In addition to directly eliminating pathogens and pathogen-infected cells, the innate immune system plays a pivotal role in priming the adaptive immune system to induce long-lived immunity against pathogens. This is accomplished via the collection and display of foreign antigens to cells of the adaptive immune system by antigen-presenting cells. Antigen-presenting cells sample the extracellular environment either by phagocytosis or endocytosis and then display epitopes of the sampled proteins on their surface, where in combination with various co-stimulatory molecules, cells of the adaptive immune system can recognize them. While many innate immune cells have some ability to present antigen, professional antigen-presenting cells, in particular dendritic cells, are highly specialized to present antigen in a manner that can drive long-lived immunity (53).

Dendritic cells (DC) function as an important bridge between the innate and adaptive arms of the immune system (54). Their main function is to take up, process, and present foreign antigens on their surface to T cells of the adaptive immune system. DCs are generated in the bone marrow and enter into the circulation and tissues in an immature form. Immature DCs patrol the body or lie dormant in tissues awaiting an infection. Because immature DCs have high endocytic activity, these cells can rapidly engulf pathogens, infected cells, or apoptotic cell debris, rapidly degrade the material, and quickly present these antigens on their surface. The engulfment of foreign antigens (integration of the danger signal described below) results in the rapid maturation of the DC, which results in decreased endocytic activity, increased migratory activity, and increased immunostimulatory capacity. The latter is accomplished via an increase in the expression of several classes of costimulatory receptors on the cell surface that are required for priming effective T-cell immunity. Thus upon maturation, the DC migrates to the lymph node where it can present the foreign material to T cells (55). Two predominant types of dendritic cells have been described, myeloid dendritic cells and plasmacytoid dendritic cells, and both types play an important role in the generation of long-lived immune responses.

Myeloid DCs (mDCs) are the principal antigen-presenting cells in the body. While macrophages are more abundant and can also capture and present antigens, mDCs

are rarer, have greater T-cell activation potential, and exclusively function to activate naïve T cells (56, 57). Given their crucial role in the generation of an effective immune response, many viruses have evolved elaborate strategies to dampen mDC activity. Many viruses selectively infect these cells, including lymphocytic choriomeningitis virus, ebola virus, dengue virus, and HIV (58–60), to eliminate these cells or manipulate their ability to induce effective T-cell immunity, thereby evading the adaptive immune response. For example, although HIV infection of mDCs remains controversial, once inside, HIV expresses an infectivity factor, named Nef, that rapidly downregulates MHC expression, thereby preventing antigen presentation to CD8 T cells (61). In addition to directly modulating presentation of antigen on the surface of mDCs following infection, many viruses perturb the expression of costimulatory molecules on the surface of mDCs, further dampening the induction of robust T-cell immunity.

Plasmacytoid DCs (pDCs) are a tiny subset (<0.5% of circulating white blood cells) that are critical for the initiation of the immune response following infection. Unlike mDCs, pDCs selectively express receptors that recognize intracellular single-stranded RNA (TLR7) and DNA (TLR9), making them uniquely poised to recognize viral infections. Once activated, pDCs are the primary producer of the inflammatory cytokines, interferon-α and -β, that detonate a highly specialized antiviral immune response. A large array of viruses can trigger the production of interferon-α and -β from pDCs including herpes simplex virus, HIV, influenza virus, newcastle disease virus, vesicular stomatitis virus, and sendai virus. However, in addition to interferon-α and -β, pDCs also produce other pro-inflammatory cytokines and chemokines that further activate the immune response and recruit additional innate immune effector cells to the site of an infection. This early cytokine cascade is critical for not only potentiating the innate immune response to the virus but also triggering the release of B cell activating and survival factor (BAFF), a key cytokine that is required for the evolution of the B cell response and generation of antibody-secreting cells (62). Clinically, elevated pDCs have been associated with reduced severity of dengue virus infection. Conversely, pDC numbers are progressively depleted in HIV infection (63), likely contributing to the compromised immunity observed in HIV-infected subjects (64).

The Complement System

In addition to innate cells, a crucial component of the innate immune response to infection is driven by a cell-free network of proteins known as the complement system. The complement system consists of a network of more than 30 proteins found in the blood that can induce the direct lysis of pathogens, the agglutination and phagocytosis of pathogens, and the chemotaxis of macrophages and neutrophils (65). Liver, monocytes, and macrophages synthesize the components of the complement system as inactive proteins that are rapidly cleaved and activated upon exposure to a pathogen. Upon activation via one of three pathways (classical, alternative, or lectin pathway), a complex cascade of cleavage events is initiated that ultimately result in the deposition of a complex of complement proteins (known as the membrane attack complex) on the surface of the target, which ultimately lead to destruction. Importantly, many of the cleaved by-products of this cascade are highly inflammatory and function to increase vascular permeability and recruit additional effector cells to the site of infection, further enhancing the innate inflammatory response.

The classical pathway is triggered by antibody binding to the target antigen. The binding of the first component of the complement cascade (C1, itself a heteropentameric complex of C1q, C1r, and C1s) to antigen-complexed antibody results in the activation of the protease activities of C1r and C1s. The active C1 can then initiate the cascade of cleavage and activation that ultimately results in the cleavage of C5 (C1 cleaves C2 and C4, which cleave C3, which cleaves C5) and the deposition of the membrane attack complex. Unlike the classical pathway, the alternative pathway does not depend on antibodies but rather is initiated after spontaneous C3 activation. Spontaneous cleavage and activation of C3 exposes a reactive thioester in C3 that allows its deposition directly onto the surface of a pathogen or cell. Upon binding to a cell, the C3 is then able to bind to additional components of the alternative pathway (including factor B and factor P), ultimately leading the cleavage of C5 and deposition of the membrane attack complex. Importantly, as the alternative pathway is initiated spontaneously, normal cells express a number of complement regulatory proteins (e.g., CD35, CD46, CD55, CD59) that can disarm the complement cascade that have been deposited on their surface, preventing unwanted pathology. However, because pathogens, including viruses, do not express these regulatory proteins and infected or dying cells have reduced expression of these molecules, the alternative pathway can rapidly eliminate infections before the production of antibodies, which are necessary to initiate the classical pathway. Finally, the lectin pathway is triggered in a manner similar to the classical pathway. However, rather than triggered by antibodies, the lectin pathway is triggered by proteins that recognize particular glycan structures (e.g., mannose-binding lectin). The binding of these proteins to the surface of cells or pathogens results in the activation of proteases similar to C1r and C1s, which in turn can activate the remainder of the cascade.

All three complement pathways have been implicated in the host immune response to many viruses, and deficiencies in complement activation have been associated with poor outcomes for many infections (66). For example, although antibody-mediated neutralization has been linked to vaccine efficacy for many clinically approved vaccines, recent data suggest that protective antibodies, like those induced by the smallpox vaccine, require the cooperative recruitment of complement to mediate their antiviral activity (67). Given the importance of complement in the control of viral infections, it is not surprising that many viruses have evolved elaborate mechanisms to evade the complement system. For example, HIV inactivates the complement system by capturing complement inhibitors (e.g., CD55 or factor H) on the virion surface, thereby protecting itself from destruction (68).

Innate Immune Recognition of Viruses

Unlike the adaptive immune system, which recognizes pathogen-derived antigens in a highly sequence-specific manner, the innate immune system recognizes microbe-associated molecular patterns (MAMPs; also known a pathogen-associated molecular patterns, PAMPs) that are associated with particular groups of pathogens. These PAMPs typically play an essential role in the life cycle of the pathogen, such as single-stranded RNA or components of bacterial cell walls, and are recognized by groups of innate immune receptors, known as **pattern recognition receptors** (PRRs).

Innate Immune Receptors

PRRs detect classes of molecules including nucleic acids, proteins, lipids, and carbohydrates that are unique to

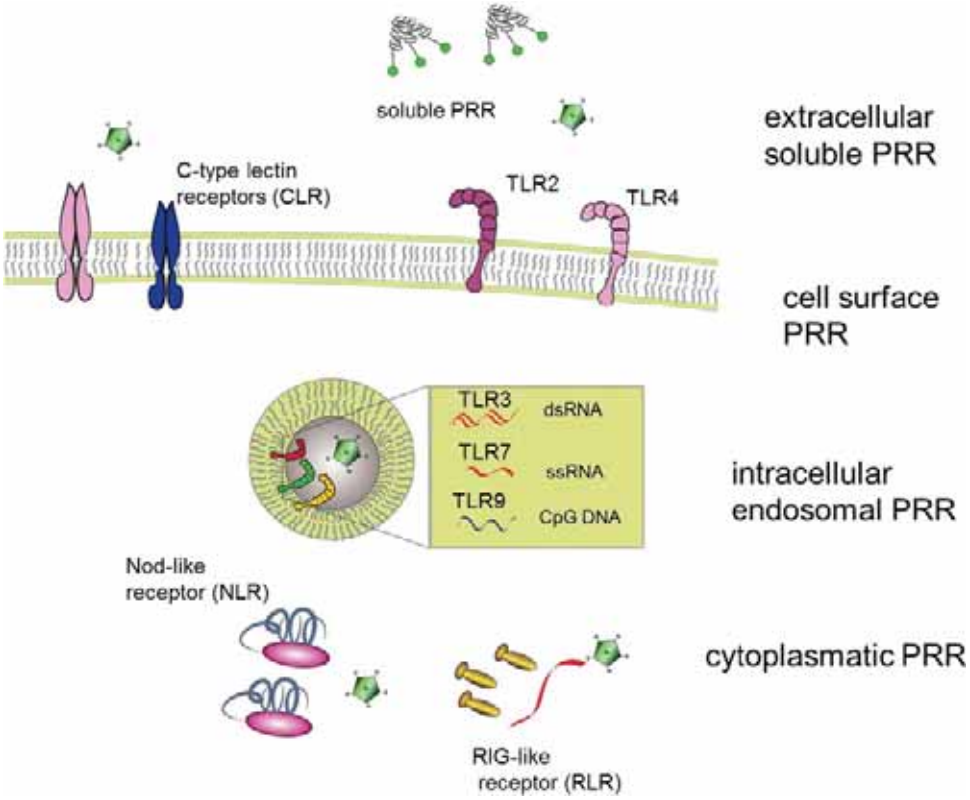

FIGURE 3 Innate immune receptors. Four types of pattern recognition receptors exist that drive a rapid response to foreign pathogens including 1) extracellular secreted receptors such as mannose-binding lectin, 2) cell surface toll-like receptors or c-type lectin receptors, 3) endosomal TLRs, and 4) cytosolic pattern recognition receptors such as RigI. The secreted and surface-bound PRRs recognize extracellular pathogens whereas the endosomal and cytosolic PRRs recognize pathogens that have entered into different cellular compartments within the cell.

particular classes of microbes. Thus, via the expression of distinct classes of PRRs, innate immune cells have the capacity to specialize in the recognition of specific classes of pathogens, contain them, and alert the immune system of infection. Importantly, many different classes of PRRs have been identified over the past two decades; these receptors not only specialize in the recognition of specific ligands but are also expressed in a highly specialized manner on and within particular immune cell subsets, greatly optimizing their function. Thus, PRRs can be located on the cell surface, within endocytic vesicles, and within the cytoplasm and can even be secreted to capture microbial products outside the cell (Fig. 3).

Toll-Like Receptors

First discovered in *Drosophila*, toll-like receptors (TLRs) are a unique class of highly conserved, type I transmembrane proteins that recognize structurally conserved motifs, including PAMPs that are exclusively expressed by pathogens and damage-associated molecular patterns (DAMPs) that are expressed on damaged cells (69). PAMPs include the structural components of bacteria (e.g., lipopolysaccharides, peptidoglycans, flagellin), bacterial DNA, and viral RNA, while DAMPs include intracellular proteins such as heat shock proteins and protein fragments. While these receptors recognize an array of different ligands, all TLRs possess a characteristic leucine-rich repeat-containing extracellular domain and a conserved Toll/IL-1 receptor cytoplasmic domain that drives cellular activation and cytokine secretion upon ligation (70).

Twelve TLRs have been characterized to date in mice (TLR1–9 and TLR11–13), of which 10 are also expressed in humans (TLR1–10). TLR14 is only found in the Takifugu pufferfish. TLR2 recognizes PAMPs from Gram-positive bacteria, including lipoproteins, lipomannans, and lipoteichoic acids. TLR3 recognizes double-stranded RNA, which occurs during replication of some viruses, and TLR4 recognizes lipopolysaccharides. TLR5 recognizes bacterial flagellin, and TLR7 and TLR8 recognize single-stranded RNA. TLR9 recognizes unmethylated DNA motifs, which are frequently found in bacteria, and the agonist for TLR10 is unknown. Because TLRs are able to heterodimerize, they can extend their individual specificities to target additional PAMPs; for example, the heterodimer of TLR2/6 can further target diacylated lipoproteins and TLR2/1 can bind to triacylated lipoproteins. Moreover, the extended range of ligands that the heterodimers can recognize is coupled to enhanced cellular functionality via the increased number and diversity of intracellular adaptors and accessory molecules that interact with the heterodimers, resulting in a further tuning of innate functionality. The cellular localization of TLRs within the cell further aids in specializing TLR function. Specifically, TLR1, 2, 4, 5, 6, and 10 are expressed on the cell surface, whereas TLR3, 7, 8, and 9 are exclusively expressed in endocytic vesicles (Fig. 3). This unique cellular localization may aid in concentrating these innate sensors at

locations within cells where their respective ligands are most likely to be detected and where the TLRs themselves are least likely to drive immunopathology. For example, TLRs3, 7–9, which recognize nucleic acids associated with pathogens, are located within endocytic vesicles. This enables these TLRs to more effectively recognize viruses or bacteria that have already infected a cell. Thus, TLR3, 7/8 and 9 play a critical role in sensing viral infections and the initiation of the antiviral immune response.

While TLRs are critical for early nonspecific innate recognition, these receptors are expressed on cells of both the innate and adaptive immune system. On innate immune cells, TLRs rapidly program innate immune activity to drive a "pathogen-appropriate" immune response. Thus, depending on the TLR or combination of TLRs triggered, the innate immune system integrates this information to discern whether a bacterium-, parasite-, or virus-specific response would be most effective. Accordingly, fundamentally different immune cascades may be deployed for the containment of bacteria (largely extracellular organisms) compared to the purging of viruses (largely intracellular organisms). Moreover, these innate immune programs additionally also qualitatively tune the evolving adaptive immune response via the secretion of cytokines or the ligation of co-receptors that provide information regarding the type of invading pathogen aimed at promoting more effective immunity. However, adaptive immune cells themselves can also express TLRs, where they likely play two distinct roles: (1) tuning the primary immune response in a pathogen-specific manner and (2) rapidly promoting more effective reactivation of memory immune responses. Thus, in the adaptive immune system, TLRs synergize with the receptors of the adaptive immune system to adjust pathogen-appropriate responses.

C-Type Lectin Receptors

Every organism is composed of four essential building blocks: protein, lipids, carbohydrates, and nucleic acids. Amino acids and nucleic acids are the most conserved across all organisms, but lipids and carbohydrates are more variable and vary wildly both in structure and composition across bacteria, parasites, viruses, and humans (71). Additionally, carbohydrates and lipids also vary extensively in human disease, such as cancer, resulting in the production of glycan-based antigens such as the carcinoembryonic/oncofetal type neoantigens (72). Thus, the immune system has evolved a unique set of innate immune receptors, known as C-type lectin receptors, that are able to specifically survey for carbohydrate-based antigenic variation. Because most pathogens are covered in unique glycans such as high levels of mannose on viruses and fungi and high levels of fucose on bacteria and helminths, C-type lectin receptors play a critical role in both host immune defense and in the clearance of aberrantly glycosylated material. Importantly, among the C-type lectin receptors, distinct cellular functions may be elicited, including endocytosis or signaling to drive distinct effector functions. This qualitative tuning is dictated by the signaling adaptors for each C-type lectin receptor and is driven by both the receptor itself as well as additional costimulatory signals that are received through other pattern recognition receptors (including TLRs). Two very broad families of C-type lectin receptors have been described, mannose receptors and asialoglycoprotein receptors.

Mannose Receptors

Humans utilize mannose widely in protein glycosylation, but few glycoproteins within our bodies are decorated with simple mannose structures or terminally manosylated structures (73). In stark contrast, oligomannose structures decorate the surface of many viruses (HIV, hepatitis C, Ebola virus, etc.) and fungi. The mannose receptor family recognizes these terminal mannoses (as well as other glycan structures) on the surface of pathogens or infected cells, and is predominantly expressed by macrophages and dendritic cells. Surface mannose receptors recognize terminal mannose, N-acetylglucosamine, and fucose residues attached to proteins and induce phagocytosis of the pathogen. Similarly, the secreted mannose-binding lectin (MBL), which recognizes mannose and fucose, cooperates with members of the complement pathway to drive the rapid endocytosis and arming of innate immune cells for pathogen destruction and inflammatory activation. Moreover, studies during the severe acute respiratory syndrome coronavirus (SARS-CoV) outbreak revealed an association of enhanced SARS-CoV infection among individuals with a polymorphism resulting in low MBL expression, thus highlighting the importance of this PRR in the control and prevention of viral infection (74, 75).

Asialoglycoprotein Receptors

Asialoglycoprotein receptors are a broad superfamily of C-type lectin receptors that are involved in the rapid clearance of glycoproteins that are not terminally sialylated (76). Receptors in this family include macrophage galactose-type lectin, DC-SIGN, langerin, dectins, and macrophage-inducible C-type lectin. These receptors collectively recognize unique glycan variants specific to distinct pathogens or improperly glycosylated host proteins, driving rapid clearance and immunological activation only in the setting of coligation with TLRs or other pattern recognition receptors.

Cytoplasmic Sensors

Because some pathogens are able to invade host cells without activating extracellular or endocytic recognition pathways, additional pattern recognition receptors have evolved to sense infection within the cytoplasm. These intracellular cytoplasmic pattern recognition receptors are able to recognize PAMPs or DAMPs via the formation of oligomers that activate rapid degradation and intracellular signaling to arm the immune response against the invading pathogen. Two broad classes of cytoplasmic pattern recognition sensors have been identified: NOD-like receptors and RIG-I-like receptors.

NOD-like Receptors

NOD-like receptors are a family of over 20 cytoplasmic pattern recognition receptors that can be broadly divided into four classes based on their N-terminal domain, NLR-A (Nod-like receptor A; "A" for acidic transactivating domain [e.g., CIITA]), NLR-B ("B" for baculovirus inhibitor of apoptosis protein repeat [e.g., NAIP]), NLR-C ("C" for caspase activation and recruitment domains (CARDs) [e.g., NOD1 and NOD2]), and NLR-P ("P" for pyrin domain [e.g., NALP1–14]) (77). NOD-like receptors recognize PAMPs and DAMPs, likely via their LRR domains, and ligand binding induces their oligomerization. These oligomers can then both activate inflammatory caspases like caspase 1, which is necessary for the maturation of proinflammatory cytokines like IL-1, and activate the signaling pathways necessary for the production of pro-inflammatory cytokines.

NODs

NODs belong to the NRLC family of NOD-like receptors and recognize peptidoglycans from Gram-negative bacteria

(NOD1) or muramyl dipeptides found in both Gram-positive and Gram-negative bacteria (NOD2). In addition to the recognition of bacterial ligands, NOD2 has also been shown to respond to several viruses, including respiratory syncytial virus, parainfluenza virus, influenza virus, and vesicular stomatitis virus (78). The recognition of these ligands results in rapid oligomerization and signaling through recruited serine/threonine protein kinases (e.g., RIP2), which ultimately result in the production of proinflammatory cytokines.

NALPs

NALPs belong to the NLRP family of NOD-like receptors, which are characterized by their N-terminal PYD domain. To date, 14 NALPs have been identified, and NALPs are activated in response to a wide range of ligands, including muramyl dipeptides (like NOD2), anthrax toxin, whole bacteria, bacterial RNA, and pore-forming toxins. Additionally, a number of viruses have been shown to activate specific NALPs, including Kaposi's sarcoma, herpesvirus, vaccinia virus, adenovirus, influenza virus, measles virus, hepatitis C virus, and HIV (78). Importantly, NALPs are necessary for the formation of several different types of inflammasomes, which are multiprotein complexes containing caspase 1 that plays an important role in the activation of the antiviral inflammatory cytokines IL-1β and IL-18.

RIG-I-like Receptors

The RIG-I-like receptor family is composed of three proteins: RIG-I, MDA5, and LGP2 (79). This family represents a unique class of cytoplasmic RNA helicases that recognize intracellular double-stranded or single-stranded RNA, resulting both in the production of interferons α and β, which functions to limit viral replication, induce the apoptosis of infected cells, recruit additional effector cells to the site of infection, and activate the inflammasome. A number of viruses have been shown to be recognized by RIG-I and/or MDA5, including Japanese encephalitis virus, hepatitis C virus, ebola virus, polio virus, and HIV (80). The critical nature of RIG-I-like receptors in antiviral immunity is highlighted by the evasion strategies developed by RNA viruses to subvert this potent antiviral activity. For example, two proteins encoded by hepatitis C virus (HCV) block a key step in the intracellular signaling cascade activated by RIG-I signaling. Similarly, the HIV-1 protease has been shown to degrade an important signaling node in the RIG-I-induced cascade.

Induced Innate Immune Responses to Infection

Following activation of PRRs, a complex network of signals leads to both the immune-mediated clearance of the PAMP/DAMP-covered material and the rapid activation of the immune system via the induced release of cytokines and chemokines. Cytokines are small proteins that are released by various cells in the body in response to cellular activation (81). Over 40 cytokines have been identified to date, and these cytokines are often classified into families based on their structure or the receptor to which they bind. Importantly, each cytokine binds to a distinct cytokine receptor, differentially expressed on the surface of cells of the body, enabling a highly specialized responses to take place depending on the cytokine cascade. Along these lines, following immune recognition of a pathogen, networks of cytokines are rapidly released by infected cells and innate and adaptive immune cells present at the site of infection. These cytokines establish the "quality" of the inflammatory milieu and both provide instructions for the elimination of the pathogen but also can signal for the recruitment of additional cell subsets that are required for enhanced pathogen clearance.

Interferons

Among the first cytokines produced following viral infection are a family of cytokines known as interferons. The interferon family plays an essential role in the earliest host immune response to infection, particularly with viruses, by placing infected cells and neighboring uninfected cells in an anti-viral state. The sensing of double-stranded RNA, which is a common intermediate in the replication cycle of many viruses, induces interferon production by infected cells. Interferon signaling in neighboring uninfected cells triggers the expression of multiple defense mechanisms aimed at interfering with viral replication, increasing antigen presentation to aid in the adaptive immune elimination of infected cells, and activating recruited adaptive immune cells (82). Three families of interferons have been described, Type I, Type II, and Type III, and these families play distinct roles in the innate response to viral infection (83).

Type I IFNs

The Type I family of interferons includes IFN-α and IFN-β (84). Type I interferons are among the first cytokines produced by an infected cell, as among others cytoplasmic nucleic acid stimulate their induction through the TBK1/STING/IRF3 pathway. While two types of IFN-β (β_1 and β_2) exist, 13 subtypes of IFN-α with different activities have been identified in humans. Virtually any cell in the body can produce Type I interferons. Importantly, type I IFN signaling cascades induce the expression of a number of host restriction factors that rapidly recognize and restrict viral infections, including the enzyme oligoadenylate synthetase, protein kinase R (PKR), members of the tripartite motif-containing protein (TRIM) family, members of the apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G (APOBEC) family, and tetherins. For example, oligoadenylate synthetase activates intracellular RNAses, including RNAse L, which degrades all RNA, both host and viral, within the cell. Similarly, activation of PKR inhibits protein translation within the cell, preventing further viral propagation. The TRIM family of proteins contains over 50 different members with distinct functions, including many with antiviral functions (85). For example, TRIM5α recognizes motifs in retroviral capsid proteins and interferes with viral uncoating, while TRIM22 inhibits transcription from retroviral promoters and blocks the assembly and budding of newly formed virions. APOBEC proteins, a family of evolutionarily conserved cytidine deaminases, drive the attenuation of retroviruses by editing viral genomes present in nascent virions, inducing their rapid degradation (86). Tetherins function to prevent the release of viral particles from the surface of cells (87) and have been linked to the inhibition of HIV budding in the absence of Vpu (88). Furthermore, tetherin has been shown to play a role in lassa and Marburg virus infection (89).

Type II IFNs

IFN-γ is the only member of the Type II family of interferons (90). While IFN-γ is thought to be more of an adaptive cytokine, released by T cells, several classes of innate immune cells, including natural killer cells, monocytes, and dendritic cells, also produce IFN-γ. IFN-γ is highly pleiotropic, inducing very broad effects, including increasing the cytotoxic

activity of NK cells, improving antigen presentation to promote more effective killing by cytotoxic T cells, increasing nitric oxide synthesis in neutrophils, promoting more class switching to more potent antibody subclasses in B cells, promoting T-cell differentiation, activating phagolysosomes in macrophages after CD4 T-cell antigen recognition, and promoting cellular adhesion and extravasation that collectively restrict, control, and promote clearance of viral infection.

Type III IFNs

The Type III family of interferons is relatively poorly characterized, although four family members have been identified, IFN-λ1–4 (91). Type III interferons are thought to play an important role in the innate response to viral infection. Many viruses have been shown to induce the expression of Type III interferons, including respiratory syncytial virus, influenza virus, Sendai virus, and HCV. While almost any cell type appears capable of expressing Type III interferons, dendritic cells, both myeloid and plasmacytoid, appear to produce the most. Unlike Type II interferons, Type III interferons induce a generalized antiviral state in cells exposed to them. This antiviral state is very similar to the state induced by Type I interferons, and in fact, many of the genes that are upregulated in response to Type I interferons are also upregulated in response to Type III interferons. One key difference, however, is that although the changes in gene expression following exposure to Type I interferons tend to be rapid and short lived, the gene expression changes following exposure to Type III interferons are slower but more prolonged. Intriguingly, receptors for Type III interferons are highly expressed in the liver, lung, and intestine, and polymorphisms in the interferon λ locus have been shown important in the response and clearance of HCV (92).

Pyrogenic Cytokines

One of the important networks of cytokines that is released in response to viral infections is a group of cytokines that have fever-inducing, or pyrogenic, activity (93). During the initial days of infection a cytokine cascade, or storm, is initiated that drives rapid and general immune activation (94). The main cytokines with these effects are IL-1, IL-6, and TNF-α, which are all released by tissue macrophages rapidly after recognition of a pathogen. Collectively, these cytokines can act directly on the brain to raise the thermoregulatory set point in the hypothalamus. The increased temperature simultaneously restricts the replication of invading pathogens, because most pathogens grow better at lower temperatures, and increased temperatures also improve immune function, as cells of the adaptive immune response are more active at increased temperatures. These cytokines also have important secondary systemic effects as they can increase the activity of the complement cascade via the activation of the acute-phase liver response, increase neutrophil production and egress from the bone marrow, and induce the migration of dendritic cells to the lymph nodes where they can stimulate the adaptive immune response. In addition to these systemic effects, these cytokines also mediate local effects, including increasing vascular permeability, activating T cells, B cells, and NK cells, and increasing the cytotoxic activity of macrophages.

Chemokines

Chemokines are cytokines that are centrally involved in driving cell movement or chemotaxis (95, 96). These proteins are structurally similar with four cysteine residues that give these proteins their characteristic structure. They are involved in immune regulation at several levels, in the homeostatic control of cell migration, in tissue development, as well as in the rapid recruitment of immune cells during infection. Importantly, chemokines act by attracting cells to the source of the chemokine, trafficking through the body along a gradient. Thus, upon infection or immunological priming, innate and adaptive cells are instructed to express particular chemokine receptors that enable them to sense particular chemokine gradients located within target tissues. These cells then leave their resident tissues and patrol the body for the highest concentration of the particular chemokine, taking up residence where the cells may perform their designated function. Following recognition of a pathogen, infected cells or neighboring uninfected cells are therefore stimulated to produce a network of chemokines to recruit additional effector cells of the innate and adaptive immune system. For example, stimulation of dendritic cells by TLR3, TLR7, TLR8, or TLR9 has been shown to induce the expression of CCL4 (also known as MIP-1β) and CCL5 (also known as RANTES). CCL4 and CCL5 recruit several players of the immune system including macrophages, NK cells and T cells. Therefore, the recognition of a pathogen can induce the expression of an elaborate network of chemokines, inducing the trafficking of a diverse range of immune cells to the site of infection, where they can mediate their protective activities.

SUMMARY

Overall, the innate immune system composed of physical barriers, commensals, effector proteins, and an array of distinct immune cells collectively act with near instantaneous speed to protect us from infection. However, beyond its role as our first line of defense, the innate immune system is also poised to set the stage for the adaptive immune system that establishes long-lived memory that enables us to rapidly resist future infections with the same pathogens.

Adaptive Immunity

The adaptive immune response is mediated by a network of lymphocytes that, following infection, acquire specificity through an evolutionarily unique set of antigen-specific receptors, providing protection from infection with the same or similar pathogen. This antigen-specific immune response is mediated by two major families of lymphocytes (T cells and B cells) that express a T-cell receptor (TCR) or B-cell receptor (BCR), respectively (Fig. 2). These adaptive immune responses are programmed through an elaborate process that begins with the generation of a large array of naïve TCR and BCR variants that then further mature, evolve, and gain enhanced specificity for pathogen-derived antigens in specialized secondary and tertiary lymphoid organs. With the influence of the innate immune response, qualitatively unique adaptive immune responses, able to provide the greatest level of pathogen-specific protection, are elicited.

T-Cell-Mediated Immunity

Introduction

T cells are lymphocytes that play a central role in cell-mediated antiviral immunity. T cells express a heterodimeric T-cell receptor (TCR; CD3) on their cell surface, which distinguishes them from other lymphocytes, including NK cells and B cells. Their name originates from the observation that a critical portion of T-cell maturation occurs in the

thymus, where T cells undergo an elaborate educational process that selects for highly effective, nonself-reactive T lymphocytes. Two major types of T cells exist, CD4 and CD8 T cells, each with distinct roles in the immune response to viral infections (Fig. 2). While CD4 T cells have been implicated as major orchestrators of the overall adaptive immune response through the release of cytokines that qualitatively tune immunity, CD8 T cells are generally regarded as the effectors of the immune system, poised to directly or indirectly eliminate infected cells.

The selection of effective CD4 or CD8 T-cell responses hinges on the "priming" process, whereby naïve T cells that recognize viral antigens on the surface of antigen-presenting cells (APCs). APCs present antigens to T cells via a unique class of cell-surface proteins known as the MHC (or Human Leucocyte Antigen (HLA) in humans) that present selected segments of digested proteins to TCRs. MHC class I generally presents cytosolic peptides to CD8 T cells, while MHC class II presents endosomal peptides to CD4 T cells. Thus, the processing, presentation, and recognition occurs through specialized machinery in distinct cellular compartments and ultimately lead to the activation and generation of highly specialized antigen-specific antiviral immune responses.

T-Cell Development

T cells originate from hematopoietic stem cells (HSCs) present in the bone marrow that populate the thymus as lymphoid progenitors (97, 98). Once in the thymus, these thymic lymphoid progenitors (or thymocytes) pass through a series of distinct selection processes that collectively ensure that only functional, nonautoreactive T cells survive and exit as mature naïve T cells (99). Roughly 4% of thymocytes survive this positive selection process giving rise to equivalent numbers of CD4 and CD8 T cells.

At the start of the selection process, thymocytes do not express any typical T-cell markers and are CD3 negative, as well as CD4 and CD8 negative (called double-negative cells). The majority (95%) of the double-negative T cells acquire an αβ TCR (100), while the remainder of T cells develop to express a γδ TCR. Antigen-specific TCR generation occurs via a process known as VDJ recombination where two (for the TCR α chain) or three (for the TCR β chain) gene segments are randomly recombined. For αβ TCRs, the process begins with recombination and expression of the TCR β chain (101, 102). Following successful rearrangement, the nascent TCR β chain can associate with a germline encoded, common pre-TCR α chain, resulting in a survival signal. Cells that fail to successfully rearrange their β-chain are eliminated. The expression of the pre-TCR then signals developing thymocytes to halt β-chain rearrangement and to undergo a proliferative burst. This results in CD4 and CD8 receptor expression and initiates α-chain rearrangement. At the end of this process a functional α chain efficiently pairs with the β chain, and the thymocyte is ready to be "tested" as to whether it is suitable as a mature naive T cell. Newly rearranged TCR-expressing T cells then undergo a positive and negative selection process aimed at selecting newly formed TCRs that are able to recognize self MHC molecules with foreign antigens (positive selection) but not self-antigens (negative selection). This process is critical to prevent the selection of self- or autoreactive T cells (103).

Positive Selection

Immature thymocytes move into the thymus cortex where thymic cortical epithelial cells present self-antigen on MHC molecules. During this interaction, only thymocytes that interact with MHC-I or MHC-II receive a "survival signal" (104, 105). Although thymocytes express both CD4 and CD8 receptors at this point, thymocytes that interact well with MHC class II molecules eventually become CD4+ T cells, whereas thymocytes that interact well with MHC class I molecules mature into CD8+ T cells (106). A T cell becomes a CD4+ cell by downregulating expression of its CD8 cell surface receptor. If the cell does not lose its MHC class II binding signal, it will continue downregulating CD8 and becomes a CD4+, single positive cell. But, if the signal is interrupted, the cell stops downregulating CD8 and switches over to downregulating CD4, and instead eventually becomes a CD8+, single positive cell. All other cells that bind either too weakly or too strongly die by "neglect" in the absence of a survival signal (107). Roughly, ∽4% of thymocytes survive positive selection process giving rise to equivalent numbers of CD4 and CD8 T cells.

Negative Selection

The surviving positively selected thymocytes migrate towards the boundary of the cortex and medulla in the thymus where medullary thymic epithelial cells (mTECs) express major "self" proteins (so called "tissue-specific self-antigens," TSAs) (108). The expression of these "self" TSAs, from a wide selection of organ specific genes, is regulated by the autoimmune regulator (AIRE). Thymocytes that bind strongly to self-antigens rapidly undergo apoptosis (via negative selection). This overall process of selection against TSAs is critical to prevent autoimmunity, and thus the activity of AIRE reduces the threat of the occurrence of subsequent autoimmunity by allowing for the elimination of autoreactive T cells that bind antigens not traditionally found in the body (109). One exception is the development of thymically derived T regulatory cells (nTregs). While T cells that receive strong positive selection signals undergo apoptotic cell death and cells that receive low signals survive. T cells that receive intermediate signals become nTregs and upregulate the Treg transcription factor FoxP3 (110), which is epigenetically modified to maintain open FoxP3 gene expression. Overall only about 2% of all T cells survive the selection process and exit the thymus as mature naïve T cells.

Triggering CD4 and CD8 T-Cell immunity

Once naïve CD4 and CD8 T cells have completed their development in the thymus they recirculate between blood and peripheral lymphoid tissues until they encounter their specific antigen. A naïve CD4 T cell recognizes specific peptides in the groove of MHC class II by its TCR (Fig. 4). The CD4 receptor binds to the β_2 domain of the MHC class II molecule D4, amplifying the signal generated by the TCR by recruiting the tyrosine kinase Lck, which is involved in the TCR signaling cascade (111). In contrast, naïve CD8 T cells recognize their specific antigen in the context of MHC class I through their TCR, while the CD8 receptor binds to the α_3 domain of the MHC class I molecule (112).

CD8 T cells play a more direct role in antiviral immunity via the recognition and lysis of virus-infected cells. Thus, MHC class I is ubiquitously expressed on all cell types, aimed at persistently sampling cytoplasmic proteins to monitor for viral infection. Conversely, while CD4 T cells can also contribute to direct cytolysis, these cells are often involved in orchestrating and shaping the adaptive immune response, qualitatively directing CD8 T-cell and B-cell immunity. Thus MHC class II is only expressed on a number of cells termed "professional" APCs that are poised to activate the

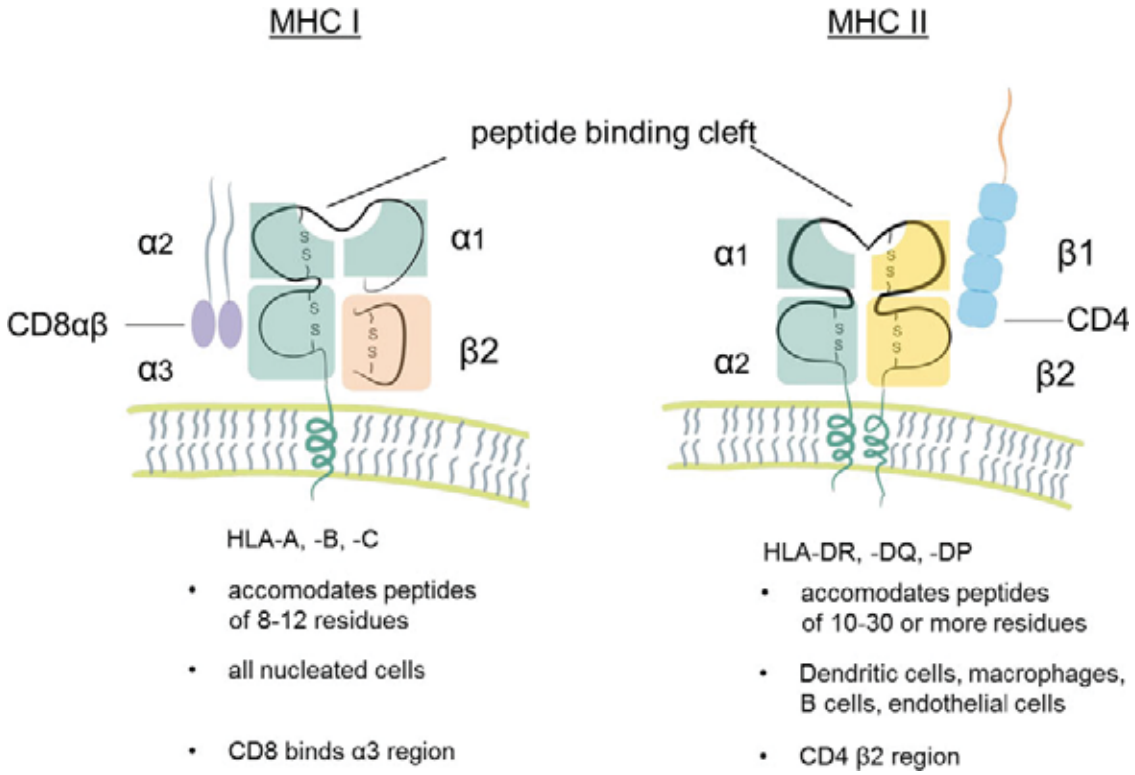

FIGURE 4 Comparison of the structure of the major histocompatibility complexes (MHC) I and II. While class I molecules are composed of a polymorphic α chain noncovalently attached to the nonpolymorphic β2 microglobulin, class II are composed of a polymorphic α chain noncovalently attached to a polymorphic β2 microglobulin. While the peptide binding cleft of MHC I is closed, allowing only little sequence variation, the binding groove of MHC class II is open, even allowing tertiary structures.

master CD4 T-cell immune regulators. MHC class II preferentially presents antigens endocytosed from the extracellular matrix.

Antigen Processing and Presentation

MHC class I and II genes, located on chromosome 6, are the most polymorphic genes of humans, providing an evolutionary advantage over many different diseases. MHC class I is composed of an α chain that consists of three domains, α1, α2, and α3. Structurally, the α1 domain sits on a unit of the non-MHC molecule β2 microglobulin (Fig. 4). The transmembrane α3 domain anchors MHC class I to the cell membrane, while the α1 and α2 domain form the peptide binding groove of the three major MHC class I genes (HLA-A, HLA-B and HLA-C), of which HLA-B is the most polymorphic allele. Each human possesses 2 sets of each of the 3 HLA class I alleles totaling up to 6 distinct HLA class I alleles that are able to present peptides to CD8 T cells.

Conversely, MHC class II is formed of two chains, α and β, each with two domains (α1/α2 and β1/β2). Each domain possess independent transmembrane domains anchoring the MHC class II molecule to the cell membrane. The α and β domains collectively form the peptide binding groove. There are three major MHC class II genes: HLA-DP (α-chain HLA-DPA and β-chain HLA-DPB), HLA-DQ (α-chain HLA-DQA and β-chain HLA-DQB) and HLA-DR (α-chain HLA-DRA and four β-chains HLA-DRB1, DRB3, DRB4 and DRB5). However, because α and β chains freely heterodimerize, heterozygous humans can have between 4 and 12 different MHC class II isoforms. Thus, there are over 10,000 potential combinations of HLA class II allele expression (113).

In addition to classical HLA alleles, a number of nonclassical MHC alleles also exist (HLA-DM and—DO) that are not exposed on the cell membrane but are involved in antigen presentation. In humans there are at least three additional nonclassical HLA class I alleles termed HLA-E, F, and G. The role of these nonclassical HLA class I alleles is less clear. HLA-E is by far less polymorphic than the other HLA class I alleles but is critically linked to NK cell recognition/activity. Yet, some virus-specific CD8 T-cell responses can also recognize HLA-E-restricted epitopes (114, 115) and have been recently linked to enhanced simian immunodeficiency virus (SIV) control and clearance in nonhuman primates vaccinated with a cytomegalovirus-based vaccine expressing SIV genes (116).

Unlike the other HLA class I alleles, HLA-F is located in the endoplasmic reticulum, and only a small amount appears on the cell surface. Interestingly, HLA-F often contains an immature oligosaccharide component and associates with at least two proteins involved in the MHC class I pathway, calreticulin and TAP. The third nonclassical HLA class I allele is HLA-G, which is thought to have an immunomodulatory role. Specifically, although HLA-G does not present peptides, HLA-G interacts with at least four different inhibitory receptors blocking the activity of NK cells, T cells, and B cells as well as some APCs. HLA-G plays an important role in pregnancy, where it inhibits the rejection of the fetal graft as a result of its expression on cytotrophoblast cells of the placenta.

In addition, homologues of MHC proteins exist, including the major histocompatibility complex class I-related chain (MIC) proteins, MICA, and MICB. Although these molecules do not bind peptides, they are selectively induced upon cellular stress, such a genotoxic stress, and serve as stress-inducible ligands for the NKG2D receptor on NK cells and on some T cells.

MHC Class I

The MHC (HLA) class I molecule presents cell-derived peptides of specific dimensions within a tight groove with closed ends. Peptides that are able to bind must form critical bonds with invariant residues in the MHC class I binding groove. Both the MHC class I pocket shape and peptide bonds restrict peptide selection to peptides that are 8 to 12 amino acids in length that are not antagonized by groove residues. Additionally, two to three pockets exist at the bottom of the groove that act as anchor residues that accommodate a highly restricted set of amino acids. Thus stabilized peptides possess a select set of anchor amino acid residues, usually located at position 2 and position 9 (117).

The importance of MHC class I peptide selection is clearest in the setting of viral evolution and escape from T-cell recognition. For example, HIV viral escape at anchor residues is often associated with T-cell evasion via a loss of T-cell recognition of the evolved virus. However, MHC class I escape is often accompanied by compromised viral replicative fitness, which has been observed for HIV and HCV (118, 119), thus viruses struggle with both advantages and disadvantages of T-cell escape to maintain infection. However, the critical importance of MHC class I targeting of viruses is most clearly illustrated by the clinical effect of particular MHC class I alleles on disease progression. For example in HIV infection the MHC class I allele HLA-B*57 has been associated with slow disease progression and control of HIV viremia via the presentation of highly conserved regions of the viral genome (120), while HLA-B*35px has been associated with rapid disease progression and, therefore, poor prognosis due to enhanced peptide presentation from variable domains of the virus (121). Similar observations have been made for MHC class I alleles in association with clearance of HBV and HCV infection.

Loading Peptides into MHC Class I Alleles

By hijacking intracellular host machinery, viruses use infected cells to replicate and generate new virions. This process produces copious amounts of foreign peptides that are processed and presented by MHC class I or class II. Most viral peptides are derived from defective ribosomal products (DRiPs) (122, 123), which are rapidly degraded forms of newly synthesized proteins. In some instances the incoming virus also brings in sufficient quantities of viral proteins for antigen presentation. However, professional APCs can also, in some instances, transfer phagocytosed virions or viral proteins to the cytosol from their lysosomal compartments, to enable viral protein processing and presentation of "externally" derived material within the "internally" produced material that is typically presented in the MHC class I pathway. This process is called "cross-presentation."

Degradation is largely mediated by a large multicatalytical protease complex called the proteasome (124, 125). The proteasome is composed of several units including a 20S protein and 2x 19S proteins on either side of the 20S that form a donut-shaped molecule with a hollow core in which proteins are degraded. The protein degradation by the proteasome is a multistep process: (1) ubiquitination and targeting, (2) unfolding and translocation, and (3) proteolysis. In the first step a series of three enzymes (E1, E2, E3) facilitate the ubiquitination of the protein targeted for degradation. However, before it is recognized by the proteasome, the target protein must be labeled with at least four ubiquitin monomers in the form of a polyubiquitin. After a protein has been ubiquinated it is recognized by the 19S protein of the proteasome and enters the 20S protein, where it contacts the proteolytic active site (step 2). Because the 20S protein is narrow the protein needs to be partially unfolded, a process called translocation, which occurs after deubiquitination. In the last step the β-subunits of the chamber of the 20S protein start the proteolysis.

Cellular chaperones (e.g., TCP-1 ring complex, TRiC) protect new peptides emerging from the immunoproteosome, preventing further cytoplasmic degradation of these peptides. Although proteasomes can generate the precise peptides presented by MHC class I molecules, many of the peptides may be longer than the MHC class I groove and are transported into the ER irrespective of groove-binding capacity. While the carboxy-terminal region of the peptides is defined by the catalytic activity of the proteasome in the cytosol, long peptides can be further trimmed by the aminopeptidases within the endoplasmic reticulum. Among the aminopeptidases, the *endoplasmic reticulum aminopeptidase associated with antigen processing* (ERAAP) has been identified as a key contributor to trimming long peptides within the ER (126, 127). Interestingly, many of the components involved in antigen processing within the immunoproteasome are rapidly upregulated by interferon (IFN). The immunoproteosome improves cleavage of polypeptides at hydrophobic residues (and decreases after acidic residues), which results in an increased binding in MHC class I residues and therefore more effective MHC presentation to alert the immune system of infection.

Peptides are continually transported from the cytosol into the ER by the transporter associated antigen processing-1 and -2 protein (TAP-1, TAP-2). In the ER newly synthesized MHC class I α chains assemble with the membrane-bound chaperone protein calnexin to retain the MHC class I molecule in a partially folded state. Once β_2 microglobulin binds to the α chain, calnexin dissociates (128) enabling the MHC complex to bind to the peptide-loading complex (PLC) including the proteins tapasin, ERp57, and calreticulin. The PLC assists in this binding event, with the peptide and MHC class I complex. The binding of the peptide releases the PLC and MHC class I can now be presented on the cell surface.

MHC Class II

While the MHC class I binding groove is closed, restricting peptide access, the MHC class II binding groove is open at both ends allowing for much greater heterogeneity in peptide length, permitting the association with peptides that hang loosely outside of the binding groove. The peptide segments that bind to MHC class II consist of about nine amino acids, which are termed the peptide binding register. The typical anchor residues of HLA class II alleles are at positions P1, P4, P6, and P9 of the peptide register and interact with residues in the pockets of the MHC molecule. Due to the variable length of the bound peptide, peptides can adopt different conformations and can even form tertiary structures on top of the HLA class II allele.

Loading peptides into MHC class II alleles

MHC class II-peptide complex assembly, peptide loading, and transport are different and slightly more complicated

than MHC class I peptide loading (129). MHC class II largely presents peptides generated in endocytic/lytic vesicles of macrophages, immature dendritic cells, B cells, or other antigen-presenting cells (APCs). Protein-derived peptides that are digested in vesicles, such as lysosomes, are transported into the ER. In the ER, premature MHC class II binding is prevented by the MHC class II-associated invariant chain (Ii or CD74) that binds and regulates MHC class II loading. Specifically, the Ii prevents peptide binding to the MHC class II molecule, and also diverts MHC class II molecules from the trans-Golgi network to an endolysosomal compartment. Here Ii is cleaved by cathepsins, in such a way that only a fragment called CLIP (class II invariant-chain peptide) remains in the MHC class II binding groove. CLIP loaded MHC class II molecules are then transported to a specialized antigen processing compartment, the class II containing compartment (MIIC). Here, the molecule HLA-DM stabilizes the empty class II molecule and prevents its aggregation, while it also catalyzes the release of the CLIP fragment, permitting the binding of other peptides to the MHC class II molecule (130, 131). It also catalyzes the removal of unstably or weakly bound peptides to allow other peptides to replace them. Within B cells, dendritic cells, and thymic epithelial cells another molecule, HLA-DO, is involved in the peptide loading of MHC class II molecules. HLA-DO serves as a negative inhibitory regulator of HLA-DM. It is unclear why HLA-DO is only found in selected cell subsets. Once MHC class II is associated with a peptide, the complex is transported to the cell surface for presentation to CD4 T cells.

Although MHC class II presents peptides from lysosomal compartments derived largely from extracellular antigens, there are exceptional circumstances when MHC class II can also present cytosolic peptides. For example, a significant number of self-peptides presented by HLA class II molecules are derived from cytosolic proteins. The most likely mechanism by which cytosolic peptides are processed for antigen presentation through the MHC class II pathway is during the natural uptake or removal of damaged organelles or proteins that are degraded in lysosomes. This process is called autophagy, the natural cellular catabolic process of degrading and recycling unnecessary or dysfunctional cellular proteins through lysosomes (132–134). However, because autophagy is increased during cellular stress, this permits cells to process more intracellular proteins, including cytosolic viral proteins present during viral infection, for MHC class II presentation to CD4 T cells. This process is critical during Epstein-Barr virus (EBV) infection of B cells, resulting in the induction of cytolytic CD4 T cells (135). Similarly, this process may also play a critical role in driving cytolytic CD4 T-cell responses against HIV-infected CD4 T cells, shown to associate with slower HIV disease progression (136). However, the fact that infected CD4 T cells can present to other CD4 T cells via MHC class II loading of viral peptides highlights a unique exception to the MHC class II-peptide presentation dogma, whereby activated CD4 or CD8 T cells can express HLA class II molecules in humans, which has not been observed in rodents. The biological significance of class II-restricted presentation by activated T cells in humans however is still unclear.

Viral Evasion of MHC

Given the selective antiviral pressure mediated by T cells, many viruses have evolved approaches to evade immune recognition through MHC peptide presentation including mechanisms that modify the maturation, assembly, and export of MHC class I and II molecules. While the mechanisms are very different, the outcome of all these evasive strategies essentially achieves the same endpoint: the downregulation of MHC class I or II molecules on the surface of infected cells and thereby reduced antigen presentation of viral proteins. Herpes simplex virus 1 (HSV-1) and cytomegalovirus (CMV) both possess a protein that specifically blocks peptide entry into the ER (137). While the ICP47 molecule of HSV-1 blocks peptide binding to TAP, CMV encodes two proteins: the US6 molecule which directly inhibits TAP, and the US3, which blocks tapasin function of the peptide-loading complex (138, 139). Likewise, adenoviruses (AdV) have evolved an E19 protein that blocks the same proteins. Conversely, Nef, encoded by HIV, binds to MHC class I, driving rapid endocytosis and thereby evading CD8 T cell recognition. While many other evasion strategies have been exploited by viruses, the wide array of evasion strategies described here provide an example of the different strategies utilized by different viruses to avoid the antiviral pressure of T cells.

Similarly, many viruses have evolved a myriad of strategies to evade CD4 T-cell immunity. Viral mechanisms that interfere with MHC class II expression mainly affect MHC class II transcription or post-translational modification. Adenoviruses and CMV both affect the signaling cascade of MHC class II transcription. In contrast, the US2 protein of CMV translocates the DRα and the DMα chain into the cytosol, resulting in proteosomal degradation. Conversely, HPV influences MHC class II viral peptide expression by blocking acidification of Ii and thus altering vesicle traffic and endocytic machinery.

Antigen Recognition

CD4 and CD8 T cells recognize their antigens through their T cell receptors (TCRs), which are the same molecules on both cell types. However, due to the differences in co-receptor (CD4 or CD8) utilization and function, T-cell subsets respond quite differently (Fig. 5). While the CD8-α interacts with the α3 portion of the Class I MHC molecule, increasing the avidity of the TCR bond, CD4 binds to β2 of MHC II, increasing the TCR signaling via the activation of the Lck pathway.

The TCR is a disulfide-linked membrane-anchored heterodimeric protein in 95% of the T cells consisting of the highly variable α and β chains, which is expressed as part of a complex with the invariant CD3 chain molecules. As mentioned above, a minority of T cells express a γδ TCR. Each of the α and β chains are composed of two extracellular domains: variable (V) region and a constant (C) region (140). The constant region is proximal to the extracellular membrane, followed by a transmembrane region and a short cytoplasmic tail, while the variable region binds to the peptide/MHC complex. The variable domain of both the TCR α-chain and β-chain each have three complementarity-determining regions (CDRs), whereas the variable region of the β-chain has an additional area of hypervariability (HV4) that does not normally contact antigen and therefore is not considered a CDR. As with antibodies, the variable region is linked to the constant region following genetic rearrangement, with additional combinatorial diversity generated in the CDR3 regions (as with antibodies, located at the junction of variable constant regions) by the insertion of one (for α chains) or two (for β chains) additional small segments. Thus, as with antibodies, most of the diversity in the TCR occurs in the CDR3 region, which typically is in direct contact with the peptide bound by the MHC molecule. The other CDR regions contact the α-helices that form the ridges

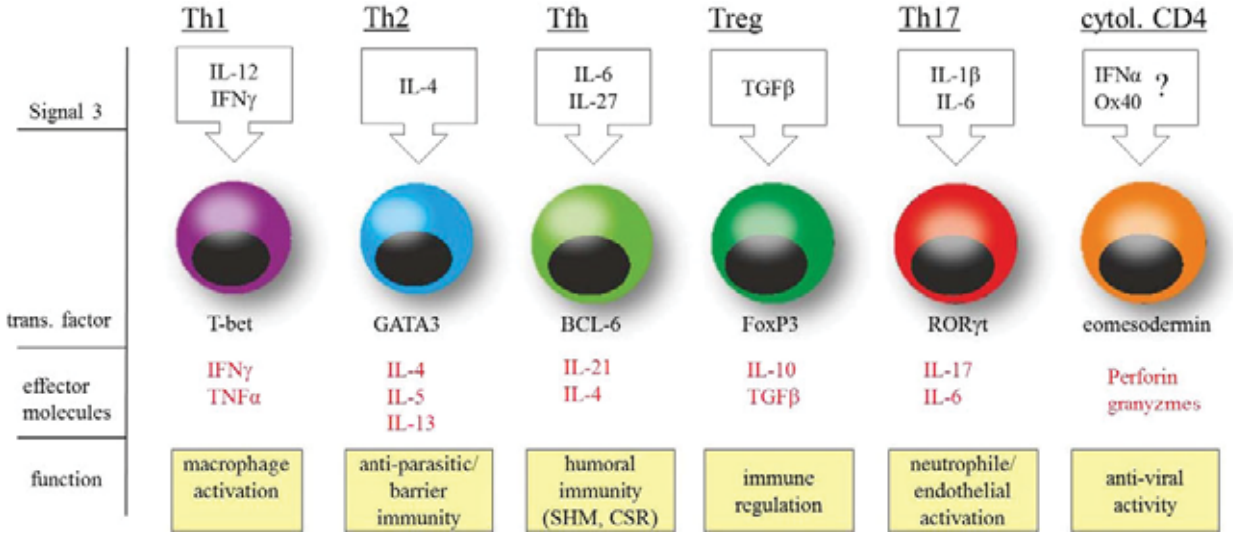

FIGURE 5 CD4 T-cell subsets. The various functional CD4 T-cell subsets are depicted, their associated main transcription factors, the cytokines they secrete, and their main effector functions.

of the MHC peptide-binding pocket. This provides the structural basis for the phenomenon of MHC restriction.

Unlike the BCR of B cells, T cells do not alter the constant region of the TCR. The TCR does not undergo somatic mutation during the course of an immune response. Thus, while the number of responding T cells increases during an immune response, the quality of the response can apparently be altered only via the recruitment of novel clonal T-cell populations (clonotypes).

Generating a T-Cell Response

Naïve T cells recirculate between lymphoid tissue and peripheral blood. During this process they bind transiently to DCs, which enables them to survey the MHCs on DC in many different organs every day. Mature DCs bind naïve T cells efficiently through interactions with LFA-1 or CD2 on the T cell with several different adhesion molecules including ICAM-1, ICAM-2, and CD58. This transient binding and surveillance is crucial to provide T cells the time to sample several MHCs on the surface of any given DC. Once the T cell encounters its antigen presented as a peptide:MHC complex on a DC, it stops circulating and the T-cell response is initiated. The activation and differentiation of naïve T cells is termed "priming." The priming of CD8 T cells usually generates cytotoxic CD8 T cells, while CD4 T cells can develop into effector CD4 T cells with a diverse array of functions. The recognition of the peptide leads to a conformational change in the LFA-1 molecule, which stabilizes the T-cell and DC interaction and the T cell forms an immunological synapse (IS) (141). The association of naïve T cells and DCs persist for several days, during which time the T cell differentiates into effector cells and proliferates. The DC feeds the naïve T cell with three distinct signals that drive T-cell activation, T-cell survival, and T-cell differentiation. The T-cell activation signal is the recognition of the appropriate peptide:MHC complex. The survival signal often comes through the ligation of CD28 with B7 molecules on the APC, which are necessary for the optimal clonal expansion of T cells. Antigen recognition and CD28 signaling triggers interleukin-2 (IL-2) cytokine secretion, which is the first step in the differentiation of T cells into effector cells (142). Furthermore, CD28 signaling also leads to a stabilization of IL-2 mRNA, which would otherwise be rapidly degraded. IL-2 is a γ-chain cytokine like IL-4, IL-7, IL-9, IL-15, and IL-21, and combinations of other γ-chain cytokines can replace the IL-2 signal but confer slightly different biological functions. For example, under certain circumstances IL-15 can have similar effects as IL-2, which is secreted by monocytes and other immune cells. The resulting effect of IL-2 or IL-15 consecutively is not identical however, and the immune system uses them in a discriminating manner to optimize T-cell activation, apoptosis, Treg activity, and memory. This autostimulation drives T-cell proliferation and allows a single T-cell clone to expand to thousands of cells all bearing the same TCR. However, these T cells also become addicted to IL-2 because removal of IL-2 results in their death, which is a mechanism exploited by Tregs to control T-cell immune responses.

The key function of IL-2 in the induction of an immune response is has been utilized in several immunotherapies that aim to suppress T-cell immunity (e.g., cyclosporin A, tarcolimus). The activation and survival signals for T cells also drive the expression of additional proteins that can modify and fine-tune the immune response. These include CD28-related proteins such as inducible costimulatory (ICOS), cytotoxic T-lymphocyte associated factor-4 (CTLA-4), or TNF-receptor family proteins such as CD27, 4-1BB, CD40L, and Ox40 (143). ICOS binds to ICOS ligand which is critical for the induction of T follicular helper (Tfh) cell development, while also regulating the expression of other cytokines such as IL-4 and IL-21. Similarly, Tfh CD40L binding to DC or B cell CD40 provides a bidirectional signal that leads to rapid and enhanced T- and B-cell maturation. In the absence of costimulatory signals, T cells can enter a state of anergy, which is critical for the prevention of autoimmunity (144). Since the thymus cannot possibly present every self-peptide, T-cell recognition of a peptide in the absence of APC activation and upregulation of costimulatory receptors renders the T cell refractory to subsequent stimulation even by professional APCs. Like many of the costimulatory signals, the signals of this interaction are bidirectional, resulting in APC anergy. While all T cells

possess a common need for IL-2 survival signaling (signal 2), the additional differentiations (3) that are required to induce various T-cell populations are distinct and therefore described in the context of their respective function.

Effector T-Cell Subsets

Cytotoxic CD8 T cells. While TCR/MHC recognition and costimulation of naïve CD8 T cells are crucial, these interactions alone do not determine the fate of specific CD8 T-cell clones. Additional signals such as the degree of antigenic activation, contact time with antigen-presenting cells, asymmetric division, and cytokine availability are forces that determine the development of CD8 T cells into phenotypically, functionally, anatomically distinct populations. Importantly, these qualitative features of the CD8/APC interaction also impact the long-term survival of the evolving CD8 T-cell response (145).

Among the key signals that drive strong CD8 T-cell responses, *in vitro* studies have shown that APC derived IL-12 can drive the proliferation and development of cytotoxic CD8 T cells. In addition, combined IL-12 and IFNα induce high levels of the cytolytic proteins, perforin and granule associated enzymes (granzymes: A-M), inside CD8 T cells that are required for *in vivo* antiviral activity. A specific set of transcription factors, called T-box transcription factors *tbet* and *eomesodermin*, are responsible for the cytolytic program of CD8 T cells (146, 147). Another transcription factor, Blimp-1, determines the effector function of CD8 T cells. Originally first identified in B cells, Blimp-1 is important in restricting and focusing the CD8 T-cell response. While more virus-specific CD8 T-cell responses are generated in the absence of Blimp-1 (148), the responses are less efficient in killing, have lower granzyme B levels, and are unable to fully differentiate into effector T cells.

The major role of CD8 T cells is to rapidly and efficiently kill virally infected cells through lysis. Target cell lysis occurs in several steps, beginning with the recognition of a foreign peptide in the groove of an MHC class I molecule on the surface of a target cell. This interaction leads to a rapid arming of the T cells that is preceded by the ligation of coreceptors and adhesion molecules including LFA and ICAM-1, that collectively create a mature adhesive immunological synapse, also called the supramolecular activation complex (SMAC) (149). The SMAC importantly creates a tight seal around the interacting TCR/MHC complexes that allows for a reorientation of the cytoskeleton and the calcium-dependent release of cytotoxic granules into the target cell. Cytotoxic granules are loaded with perforin, granulysin, and granzymes within lysosomes coated with lysosomal-associated membrane protein (LAMP)-1/-2 (CD107a/b). Perforin acts as a delivery molecule, poking holes in the target cell, through which granzymes and granulysins can enter. The granzymes trigger apoptosis in the target cells by activating caspase 3 involved in caspase proteolytic activity, leading to the activation of the caspase-activating desoxyribonuclease (CAD) (150). This nuclease acts as a DNAse and degrades the target cell DNA. Furthermore granzymes also activate the BH3 interacting domain (BID) that disrupts the mitochondrial outer membrane, driving cellular apoptosis. Additionally, beyond direct cytolysis, a small fraction of CD8 T cells can also kill their targets via the induction of apoptosis through the interaction of Fas-ligand (Fas-L) on the CD8 T cells and Fas on the target cell.

In addition to their role in cytolysis, CD8 T cells also secrete several proinflammatory antiviral cytokines, including IFNγ and TNFα, which indirectly contribute to the antiviral host response. As mentioned above, IFNγ can regulate MHC class I expression, activation of TAP, increase lysosomal activity, upregulate TRIM5α, APOBEC, and tetherin. TNFα is also a chemoattractant and plays an important role in the activation of macrophages that may contribute to the clearance of apoptotic debris. However, TNFα can also bind to TNFR-I expressed on some target cells and thereby drive direct killing.

In addition to cytotoxic CD8 T cells, a small subset of CD8 T cells evolve with regulatory T-cell activity. Regulatory CD8 T cells are characterized by the expression of the transcription factor FoxP3 and express CD122 and are CD28 low. While these cells are largely involved in limiting autoimmune disease by the reduction of T-cell proliferation by secretion of IL-10, for example, they have also been implicated in limiting chronic immune activation in chronic viral infections caused by viruses such as HCV, HIV and EBV to prevent to immune-induced tissue damage (151, 152).

CD4 T-Cell Help for CD8 T-Cell Immune Responses. Although potent primary CD8 T-cell responses can be induced in the absence of CD4 T-cell responses, the establishment of long-lived, functionally robust memory CD8 T-cell activity requires the presence of CD4 T-cell help (153). Specifically, CD8 T-cell responses generated in the absence of CD4 T-cell help may remain in an effector-like state, associated with the constitutive expression of the transcription factor T-bet (154). These "unhelped" CD8 T cells develop a spectrum of functional defects such as compromised cytokine-producing capacity and diminished proliferative activity, often referred to as exhaustion (155). However, the precise pathway by which CD4 T cells help program CD8 T-cell memory is not fully understood. In addition, it has been shown that secretion of CCL3 and CCL4 by CD4 T cells can promote CD8 T-cell migration towards DCs (156). Moreover, CD4 T cells also appear to be essential in "licensing" DCs via CD40L-CD40 interactions to provide optimized signals to CD8 T cells during the priming process (157). Third, CD4 T cells also secrete IFNγ, CXCL9, and CXCL10 required to direct CD8 T cells to the site of infection, which has been shown to play a role in HSV infection (158).

Furthermore, CD4 T cells also promote more effective CD8 T-cell activity directly via the secretion of both IL2 and IL21 (159, 160). While IL2 provides growth, survival, and differentiation signals to responding CD8 T cells, IL21 has been implicated in increasing the antiviral potency of CD8 T cells and increasing the production of granzyme and perforin. The importance of IL2 and IL21 signals in driving more effective T-cell immunity has been shown in many human viral infections, including HCV and HIV (161, 162). Indeed, in LCMV infection of mice, it has been demonstrated that CD8 T cells cannot control infection in the absence of IL21 secretion by CD4 T cells (159, 160).

CD4 T Cells. While the initial steps of antigen recognition by CD4 T cells through the TCR:MHC class II is similar to that of CD8 T cells, CD4 T-cell differentiation following activation is more complex because of the large array of distinct differentiation pathways that exist for this subset of T cells. Interestingly, CD4 T-cell differentiation is tightly linked to APC interactions. APCs not only present foreign antigens to CD4 T cell, but also produce cytokines and express costimulatory molecules that ultimately program CD4 T-cell differentiation, aimed at shaping CD4 help in a pathogen- and tissue-specific manner. Thus APCs that

integrate information through their innate immune receptors can then selectively skew the lineage commitment of CD4 T cells. There is evidence, however, that the commitment of CD4 T cells to one effector function is not necessarily set in stone and that under certain circumstances CD4 T cells can revert to a different CD4 T-cell effector function, suggesting that there is some plasticity in lineage development (163). Yet among the CD4 T-cell lineages, at least seven distinct T-cell subsets have been identified, each defined largely on the basis of the transcription factors that define their lineage and the cytokines that they secrete (Fig. 5).

TH1 CELLS. Th1 cells are induced in the presence of DC-derived IL-12 and DC or NK cell-derived IFNγ. The signature cytokine of Th1 cells is IFNγ, but they also secrete IL-2 and TNFα. Th1 driven-IFNγ secretion further potentiates the Th1 profile, and autocrine IL-2 secretion promotes effective T-cell proliferation. Th1 cells are further characterized by the expression of the transcription factor t-bet (164). While Th1 cells are mainly involved in the control of intracellular/intravesicular bacterial infections, almost all viral infections induce a strong Th1 CD4 T-cell response. Indeed, IFNγ and TNFα secreted by CD4 Th1 cells potentiates CD8 T- cell immunity and contributes importantly to shaping the antiviral milieu. For example, it has been demonstrated that influenza virus infection is less severe in the presence of pre-existing Th1 CD4 T-cell response (165), and HCV and HIV disease outcomes are improved in the presence of broad virus-specific Th1 CD4 T cells (166, 167).

TH2 AND TH9 CELLS. The absence of IFNγ signals and the presence of IL-4 drives Stat6 expression and the development of Th2 CD4 T cells. Stat6 specifically induces the expression of the main Th2 transcription factor GATA3, which is responsible for the activation and expression of Th2 cytokines including IL4, IL5, and IL13 (168). These cytokines further upregulate GATA3 expression and thus reinforce the Th2 lineage commitment bias. While the initial source of IL4, required to drive the initial lineage commitment is still unclear, basophils, eosinophils, and mast cells can all produce IL-4 and have all been implicated as potential catalysts if activated soon after infection. Critically, Th2 cells are important for the immune response against large extracellular pathogens and may play a role in the development of antibody responses against some viral infections. Similarly, Th9 cells are directed against large extracellular organisms and in particular against helminths. This newly described subset is induced in the presence of IL4 and IL9 and characterized by the expression of the transcription factor PU.1 and cytokine secretion of IL-9 (169). Th9 cells have no known role in antiviral activity.

T FOLLICULAR HELPER CELLS. An important component in the antiviral immune response is the humoral immune response, mediated by B cells. A specialized subset of CD4 T cells provide the help and instructions required to generate a robust B-cell response upon infection, the T-follicular helper (Tfh) cells. Tfh have a key role in the formation of germinal centers within lymph nodes, the affinity maturation of B-cell response, and the establishment of memory in the antibody responses (170). Tfh cells are the only subset of CD4 T cells that are defined based on their location within the B-cell follicle; although the cardinal cytokines produced by Tfh cells is IL-21.

The differentiation process of Tfh cells occurs in three phases: (a) initial differentiation towards the Tfh-lineage, (b) migration into B-cell follicles where the cells interact with B cells, and finally (c) maturation within germinal centers. In the presence of DC-derived IL-6, IL-27, and IL-12 (in humans), naïve CD4 T cells upregulate the transcription factor BCL-6 and cMaf, which drive the expression of the chemokine receptors CXCR5, ICOS, and IL-21. Tfh cells now follow the chemokine gradient CXCL13 and reposition themselves at the B-cell/T-cell border of the B-cell follicle. Here, interaction with the cognate antigen presented on the B cell drives the further development of Tfh cells. Tfh cells and B cells engage in a bidirectional interaction involving an array of receptors including CD40-CD40L, ICOS-ICOSL, SLAM receptor family, PD1- PDL1, and others to tune B-cell activity. During this process Tfh cells further develop into germinal center (GC) Tfh cells that secrete IL21 and express the transcription factor Bcl-6.

Potent, long-lived, antigen-specific antibody responses hinge on the evolution of an effective GC reaction that enables the selection of high affinity B-cell responses to target foreign antigens. Within the GC, Tfh cells instruct B cells to undergo somatic hypermutation (SHM) that drives affinity maturation of the antibody response. CD40L and IL21 are required for B-cell survival, proliferation, class switching, and differentiation into memory B cells. Second, they activate the activation-induced deaminase (AID) enzyme that drives both SHM and class switch recombination (CSR).

TH17 CELLS. Under specific inflammatory conditions, a subset of IL17-producing CD4 T cells, Th17 cells, are induced that regulate neutrophils, epithelial, and endothelial cells (171). Th17 cells are induced in the presence of IL6, TGFβ, and IL1β, which drive the upregulation of the transcription factor RAR-related orphan receptor γ (RORγ). Th17 cells play a critical role in the maintenance of the gastrointestinal tract microbial flora and control of bacterial infections, but their role in viral immunity has been disputed. However, while these cells may not contribute to antiviral immunity directly, Th17 CD4 T-cell responses play a critical role in dampening the immunopathology associated with HIV, CMV, HCV, and likely other viral infections. Specifically, following the extinction of nearly all mucosal CD4 T cells following acute HIV infection, elevated levels of Th17 cells are associated with enhanced gut epithelial integrity, reduced immunopathology, reduced microbial translocation, and therefore lower systemic immune activation. Additionally, Th17 cells may also contribute to antiviral immunity via the maintenance of the microbiome that indirectly shapes the immune response (172).

T REGULATORY CELLS. Regulatory T cells (Tregs) are a suppressive subset of CD4 cells important for the regulation of immune responses and a direct opponent of Th17 cells (173). Two types of Tregs have been described: (1) naturally occurring Tregs that are generated in the thymus (tTreg) with a TCR repertoire biased towards self-peptides, and (2) induced Tregs that emerge at later stages of infection (iTreg). Thymus-derived Tregs require CD28 costimulation, TCR engagement, and γ-chain cytokines, such as IL2, critical for the expression of FoxP3. However, IL-15, a second γ-chain cytokine, can also drive Treg generation. In contrast, iTregs are induced following the release of TGFβ and IL2.

Tregs are characterized by the demethylation of the Treg-specific demethylated region (TSDR) in the FOXP3 locus, high expression of the IL2 receptor (CD25), and low expression of the IL7 receptor (CD127). High IL2 receptor expression permits Tregs to soak up most of the IL2 in the

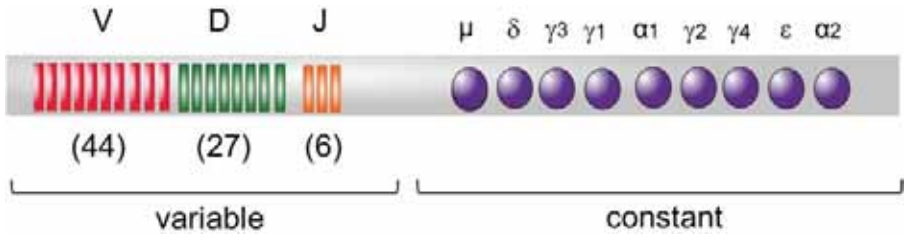

FIGURE 6 The IgH locus. The human immunoglobulin locus consists of two regions that give rise to either 1) the variable domain (Fab) or the 2) the crystallizable/constant domain (Fc). The Fab is generated following the rearrangement and selection of a V, a D, and a J segment that are assembled and tethered to the Fc region, which is also selected following gene rearrangement and selection of a single constant gene segment. Gene rearrangement requires the cutting out of excess DNA and religation of DNA ends, generating irreversible changes in the IgH locus.

peripheral circulation, thereby competing with newly primed or memory T cells for survival factors. However in addition to this indirect regulatory activity, Tregs also secrete IL10, TGFβ, and IL33, which directly act to reduce inflammation and immune activation. Interestingly, while the role of Tregs in antiviral immunity is unique to each virus, Tregs play a key role in HSV infection in blocking virus-specific CD8 T-cell mediated immunopathology and directly blocking excessive tissue immunopathology.

CYTOLYTIC CD4 T CELLS. Despite the dogma that CD4 T cells are "helper" cells, a novel subset of CD4 T cells were recently described that mediate direct antiviral effects. The conditions under which CD4 T cells upregulate cytolytic functions are not fully understood. Yet in mice, it has been described that high levels of IFNα or the ligation of Ox40 and 41BB can drive the induction of a cytolytic CD4 T-cell program similar to that of CD8 T cells. Cytolytic CD4 T cells have a more terminally differentiated phenotype, with high expression of CD57 and KLRG1, but their profiles are distinct from Th1 cells (174). Cytolytic CD4 T cells seem to be less potent than CD8 T cells, but kill virally infected cells in a similar fashion as CD8 T cells. Importantly, cytolytic CD4 T cells have been implicated in protective immunity against several human viral infections including influenza, EBV, HCV, and HIV (175).

Humoral Immunity

Introduction

With the observation that immunity could be transferred using cell-free blood components the importance of antibodies discovered (176). It was postulated that the humoral immune system is able to generate an infinite number of antibodies with unique specificities that provide long-term protection from reinfection.

B Cells

Cells are lymphocytes that develop in the bone marrow from a common lymphoid progenitor. While still in the bone marrow, the cells undergo VDJ recombination, resulting in the rapid rearrangement of a set of genes in the variable region of the immunoglobulin, encoded in the immunoglobulin heavy (chromosome 14) and light (κ-chain chromosome 2 or λ-chain on chromosome 22) chain loci (Fig. 7). During the rearrangement of the BCR, the heavy chains are rearranged to contain 1 of 44 different V-gene segments, 27 different D-gene segments (similar to TCR β chains, only the heavy chain contains D-gene segments), and 6 different J-gene segments. The random selection of distinct segments, linked to the stochastic and random repair mechanisms that bring segments together, gives rise to an enormous diversity within the B-cell repertoire. Importantly, failure to select segments that produce a functional BCR or that generate autoreactive antibodies results in the death of the B cell. Conversely, successfully rearranged B cells can exit the bone marrow as pre-B cells once they begin to express their immunoglobulin as a transmembrane IgM receptor and IgD receptor.

Similar to T cells, B cells circulate through the blood in a resting inactive state, either as antigen-naïve B cells or antigen-experienced memory B cells. However, upon encountering their cognate free-floating or APC-presented antigen through their BCR, a unique transcriptional program is turned on in the B cell, driving B-cell survival, proliferation, maturation, and in some instances terminal differentiation into antibody-secreting cells, which occurs inside the GC. However, like T cells, BCRs collaborate with additional coreceptors to tune B-cell activation, including CD21, which tunes the activation threshold of the BCR.

Both T-dependent and T-independent B-cell responses can be induced during an immune response. T-dependent antigens largely consist of protein antigens that are processed by B cells after they capture antigens with their BCRs from a specialized set of APCs, called follicular dendritic cells (FDCs). B cells process antigens and then present them on MHC class II to CD4 Tfh cells in the lymph nodes. Following Tfh recognition of peptide-MHC complexes on B cells, T cells deliver survival and activation signals to B cells by the ligation of coreceptors (CD40L) and the release of cytokines like IL-21 and IL-4, which further drive B-cell proliferation and survival. B cells that fail to present antigens recognized by CD4 Tfh perish due to a lack of survival signals. Activated B cells differentiate into activated memory B cells that cycle within the GC where they undergo extensive clonal expansion, class switch recombination, and affinity maturation to give rise to optimized BCRs that recognize and clear the pathogen with greater efficacy (Fig. 8). This extensive maturation process gives rise to hypermutated high-affinity antibodies that are thought to be critical in the evolution of neutralizing antibodies that prevent many viral infections. Additionally, through interactions with Tfh, successful memory B cells receive signals to exit the GC as long-lived memory B cells or bone-marrow destined plasma cells that secrete copious amounts of antibodies for life. In contrast, T- independent antigens are largely polysaccharides that induce B-cell responses in the absence of T cells through extensive BCR cross-linking (largely by repeating antigens on polysaccharide bacterial walls) and in conjunction with the activation of danger signals such as TLRs. While T-

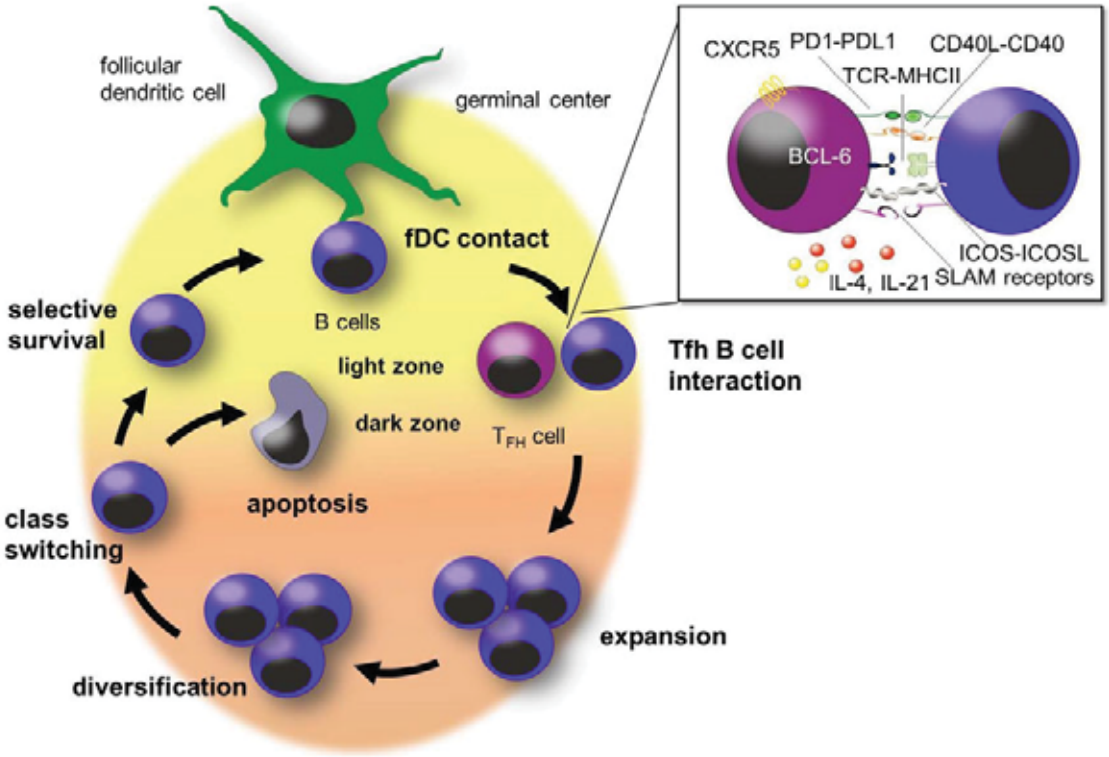

FIGURE 7 The germinal center reaction. The germinal center reaction begins with an initial contact between B cells and cognate Tfh cells through an array of different receptors such as PD1-PDL1, CD40-CD40L, ICOS-ICOS-L, and TCR-MHCII. This promotes extensive proliferation of antigen-primed B cells. The GC cycle is thought to form two microanatomically distinct regions: the T-cell zone-proximal dark zone (which contains proliferating centroblasts) and the T-cell zone-distal light zone (which contains centrocytes, follicular dendritic cell [DC] networks and antigen-specific gcTFH cells). The expansion of antigen-specific B cells in the dark zone is accompanied by B-cell receptor (BCR) diversification through somatic hypermutation and class switch recombination. Loss of binding through this process leads to apoptosis, and only the best "binders" receive positive signals and enter the cycle again until they leave the GC as plasmablasts or memory B cells.

independent responses are lower affinity and are associated with bacterial or parasitic pathogens, these responses are induced rapidly and may play a critical role in the early recognition and control of highly glycosylated viral glycoproteins, including HIV and Ebola virus envelope glycoproteins.

Importantly, antibodies circulating in the blood are produced by either long-lived plasma cells or short-lived plasmablasts (177). While long-lived plasma cells establish lifelong residence in the bone marrow, where they are able to secrete over 2,000 antibodies a second (178), short-lived plasmablasts are only generated immediately after B-cell

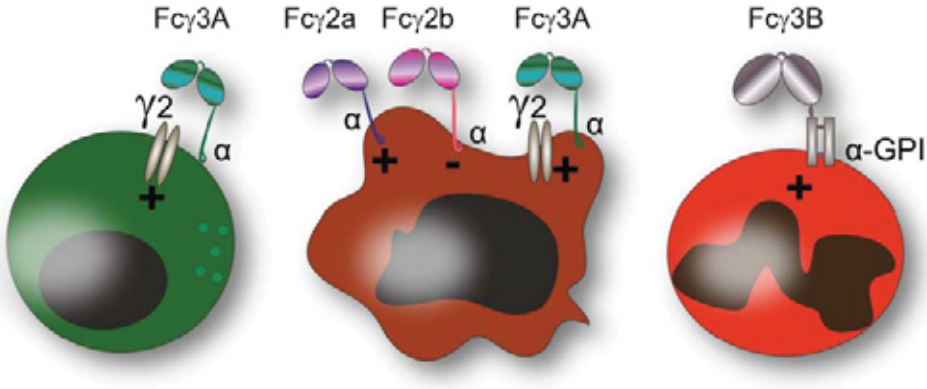

FIGURE 8 Distinct Fc-receptor expression profiles on distinct innate immune cells. Differences in Fc receptor expression profiles on distinct innate immune cell subsets enable these cells to respond to antibody-opsonized material differently to promote control and clearance of pathogens. Specifically, while NK cells and neutrophils largely express a single Fcγ-receptor, FcγR3a, on NK cells and FcγR3b on neutrophils, macrophages express nearly all Fc receptors including the two activating FcγR2a and FcγR3a receptors, as well as the sole inhibitory FcγR2b receptor.

activation and differentiation and are aimed at rapidly generating large waves of antibodies to contain and/or clear the infecting pathogen.

Antibodies

Antibodies consist of two antigen-binding domains (variable domains, Fv) and a constant domain (crystallizable domain, Fc). The presence of two identical variable domains ensures higher avidity binding to the antigen of interest, and the Fc domain provides instructions to the innate immune system on how the targeted antigen should be destroyed.

Antibody Function

For most of the clinically approved vaccines, the generation of antibodies able to block, or neutralize, the pathogen are considered the correlate of protective immunity. This neutralizing antibody property is mediated by antibodies that can block key infectious units on the pathogen (i.e., viral envelope proteins) or opsonize the pathogen in such a way that infection may be sterically hindered. However, beyond neutralization, antibodies mediate a remarkably broad array of additional functions, including the classically defined recruitment of innate immune responses. These include antibody-dependent cellular cytotoxicity (ADCC) by the recruitment of NK cells, antibody-mediated cellular phagocytosis (ADCP) by the recruitment of macrophages or neutrophils, and complement-mediated killing by the recruitment of the classical complement pathway.

Antibody Evolution

While the antibody that is produced early in the immune response functions to immediately protect cells from subsequent waves of infection and begin to eliminate the pathogen, B cells have the capacity to undergo additional genomic changes to produce antibodies with significantly higher affinity for pathogens. This **affinity maturation** occurs in the germinal center and results in the generation of waves of lineages of B cells that produce closely related antibodies with ever increasing affinities. Importantly, both the Fab and Fc domains of the antibodies can be independently optimized.

Somatic Hypermutation

During affinity maturation of B-cell responses, the variable domain is diversified and optimized via a process known as **somatic hypermutation**. The GC is a site of rapid B-cell proliferation, and during this proliferation the immunoglobulin locus undergoes a high degree of mutation, typically at rates that are over a million times greater than the rates seen in other genes. Although the genes and regulatory signals involved in controlling somatic hypermutation have not been completely identified, several components, including the DNA editing enzyme activation induced cytidine deaminase (AID) and several DNA structural elements, have been shown to be necessary for efficient somatic hypermutation. Given the size of the variable region of the antibody and the mutational rates, somatic hypermutation typically results in the acquisition of a single amino acid change per cell division, while the B cell remains in the GC. Coupled with the rapid cell division that B cells undergo in the GC (dividing every 6 to 8 hours), this results in the rapid accumulation of additional mutations in GC B cells. Importantly, because antigen-loaded APCs are present in only limited numbers in the GC, each subsequent wave of somatically hypermutated B cells must compete for binding to antigen and the resulting stimulatory signals (Fig. 8). B cells in which mutations that negatively impact antigen binding are rapidly eliminated via apoptosis because they can no longer compete with other B cells for antigen binding. Not surprisingly, GCs are sites of massive apoptosis, and these apoptotic B cells are cleared by lymph node–resident macrophages. However, when mutations that increase affinity for a target antigen are introduced, the resulting B cells are strongly positively selected, resulting in the outgrowth of progeny cells with increased affinity for the antigen of interest. These progeny cells can, in turn, undergo further somatic hypermutation, increasing the affinity for the antigen even further. Following multiple rounds of B-cell selection and somatic hypermutation, large clonal repertoires of B cells that target the pathogen with increased affinity are generated, and these cells can exit the GC to become antibody-secreting cells or memory B cells. Importantly, while affinity maturation is required for the development of all protective antibodies against all viral infections, neutralizing antibodies against HIV emerge only in a fraction of infected individuals because of the unusual characteristics of the antibodies that are able to protect against this rapidly evolving viral pathogen.

Constant Domain (Fc) Diversification

While somatic hypermutation diversifies the variable domain of the antibody, increasing the affinity of the antibody for antigen, the constant (Fc) domain of the antibody can also undergo diversification. This constant domain diversification is achieved via two modifications to the antibody Fc domain: (1) selection of one of four distinct Fc isotypes (IgM, IgG, IgA, and IgE) or one of four IgG subclasses (IgG1, IgG2 IgG3, IgG4), and (2) variation in the glycosylation of the antibody in the Fc domain. These modifications alter the affinity of antibodies for various classes of innate immune receptors (e.g., Fc receptors on the surface of all innate immune cells or complement), thereby altering the bioactivity of the antibodies against the infecting pathogen.

Class Switch Recombination

Although the variable and constant (Fc) domains of the antibody have distinct functional roles, their diversification is tightly regulated and coordinated through a common network of signals. For example, the enzyme, AID, is critically involved in somatic hypermutation of the variable domain, as mentioned above, but it is also critical in CSR that enables a B cell to select for different antibody isotypes and subclasses, with each isotype and subclass possessing different functional activities.

Upon B-cell activation, naïve B cells, which express an IgM/IgD BCR, can select a different subclass or isotype by the directed cleavage and recombination of constant region segments in the *IGH* locus (179). In the human *IGH* locus, the isotypes and subclasses are arranged in a particular sequence: IgM, IgD, IgG3, IgG1, IgA1, IgG2, IgG4, IgE, and IgA2 (Fig. 7). Each subclass and isotype gene segment is preceded by a unique switch region, which is the target of the recombination machinery during CSR. When a particular isotype or subclass is selected, the recombination machinery loops out and permanently deletes all of the intervening gene segments between the variable domain and the targeted constant domain sequence. For example, when the B cell transits from IgM/IgD to IgG2, all the gene segments encoding IgM/D/G3/G1 are all spliced out of the *IGH* locus in that specific B cell. In this example, while this particular B cell can never go back to produce an IgG3

antibody, it does still retain the capacity to further produce IgG4, IgE, or IgA2 antibodies.

CSR is a highly regulated process controlled by cytokines and helper signals (180). While the precise signals are unknown, IL-4 may selectively induce CSR to produce IgG4 and IgE (181, 182), whereas IL-10 induces CSR towards IgG1 and IgG3 (183, 184). Moreover, beyond cytokines alone, IgG subclass selection varies by infection and is related both to the inflammatory state induced by a pathogen and the location of the pathogen (e.g., intra- vs. extracellular) (185). Along these lines, HIV-specific IgG3 antibodies have been proposed as biomarkers of acute HIV infection, because of their enrichment during the first few weeks of infection (186). IgG3 antibody levels decline quickly over the first few weeks of infection and are rapidly followed by a robust wave of IgG1 responses that dominate the chronic phase of the disease (187). Most of the antibody responses to viral antigens appear to be dominated by IgG1 and IgG3 antibodies, and IgG3 has been shown to mediate most effective virus neutralization among the IgG subclasses (188), likely related to the long hinge region found in IgG3 antibodies (189). Moreover, the protective nature of IgG3 responses has been repeatedly observed against intracellular pathogens including malaria (185) and RSV (190). However, the importance of IgG3 in antiviral immunity was most clearly illustrated in the first moderately protective HIV vaccine trial, RV144 (191), which pointed to a critical role for IgG3 responses in reducing the risk of HIV acquisition (192, 193).

The order of the antibody subclass and isotypes is likely not coincidental but potentially evolutionarily programmed to allow for the selection of the most functional antibodies (e.g., IgG3 and IgG1) early in an immune response, followed by less inflammatory antibody subclasses in subsequent waves of the immune response (e.g., IgG2 and IgG4). For example, within the IgG antibodies, affinity for FCGRs, and therefore immune function, follows the same order as is encoded within the *IGH* locus with IgG3 harboring the highest affinity for FCGRs, then IgG1, then IgG2, and finally IgG4, which demonstrates negligible FCGR-mediated biological functionality. This enables new B cells to select the most functional antibody subclass earliest in an immune response and switch these out if they are ineffective.

FC GLYCOSYLATION. Although all antibody isotypes and subclasses are glycosylated, the functional consequences of IgG glycosylation have been the most well characterized. All IgG antibodies are glycosylated at a single asparagine residue (N297) within the CH2 domain of the Fc domain, and modulation of the antibody glycan rapidly and potently alters the inflammatory profile and effector functions of the antibody (194). This complex, N-linked glycan, consists of differing levels of four sugar moieties (galactose, sialic acid, fucose, and N-acetylglucosamine) attached to a biantennary core glycan structure. Particular glycan structures have been shown to differentially affect the binding of the antibody to various innate immune receptors, thereby modulating the functional activity of the antibody. For example, antibodies in which the glycan lacks additional galactose residues (known as G0 antibodies) have been associated with increased inflammation in HIV (195, 196) and chronic autoimmune conditions (197) and are known to drive enhanced complement binding and activation (198). In contrast, the presence of higher levels of galactose provides the scaffold for the addition of terminal sialic acid residues, which have been shown to have antiinflammatory activity via binding to lectin-like receptors (199). Furthermore, changes in the fucose and N-acetylglucosamine content play a critical role in modulating antibody effector function, whereas a lack of fucose (200) or the addition of the N-acetylglucosamine (201) increases ADCC activity. Yet, beyond these well-characterized glycan structure:function relationships, over 30 different glycan structures have been identified in naturally produced antibodies, each with the theoretical capacity to drive distinct effector functional profiles.

Fc Receptors

All innate immune cells express Fc receptors, which allow antibodies raised by the adaptive immune system to provide these cells with specificity to kill pathogens or infected cells. In humans, Fc receptors (FcRs) exist for individual antibody subclasses including FcαR (IgA), FcεR (IgE), and FcγR (IgG), with multiple family members belonging to each class of receptors. The broadest family of Fc receptors include the FcγR family, with six different FcγRs identified in humans. These FcγRs having various affinities for IgG (high-affinity FcγRI, and low-affinity FcγRII and FcγRIII), with several variants among the low-affinity receptors, including an activating FcγRIIA, the inhibitory FcγRIIIB, a rare activating FcγRIIC, a transmembrane-activating FcγRIIIA, and a GPI-anchored FcγRIIIB protein. Moreover, within the activating FcγRIIA and FcγRIIIA receptors, polymorphisms exist that confer increased affinity to IgG molecules, including the 131H/R *FCGRA* variants (the H variant has higher affinity) and the 158F/V *FCGR3A* SNP (the V variant has higher affinity) recognized for their ability to affect the clinical efficacy of monoclonal therapeutic intervention strategies (202). Importantly, the expression of these receptors is relatively restricted among innate immune cell subsets, providing a level of regulation of the specific innate immune effector cells that are able to respond specifically to antibody targets. For example, FcγRIIIA is expressed nearly exclusively on NK cells, whereas FcγRIIIB is expressed predominantly on neutrophils. Conversely, monocytes, macrophages, and dendritic cells express FcγRIIA and can upregulate FcγRIIIA expression upon activation. The expression of FcγRIIB is more restricted and is highly expressed on B cells and dendritic cells, aimed at regulating the activity of these cells.

Interestingly, in the context of infections, FcγR polymorphisms have been linked to both susceptibility and resistance to several infectious diseases (203). Indeed, differences in FcγRIIIA polymorphisms have also been linked to differential risk of infection following vaccination, including elevated risk of infection in the low-risk vaccine category in the HIV vaccine trial, VAX004, in which the 158VV FcγRIIIA genotype was associated with enhanced risk of infection (204). Yet, despite the effects of these polymorphisms, FcγR expression is tuned rapidly during an immune response to increase expression on innate immune cells (205). These rapid changes allow innate immune cells to respond instantaneously to pathogen-specific antibodies to drive rapid clearance and control of infection. Although neutralizing antibodies, able to block viral infection, are largely considered the correlate of protection against most viral infections, non-neutralizing, Fc-mediated antibody functionality also contributes to antibody-mediated control against West Nile virus (206), HIV (207), and ebola virus infections (207). In some instances, however, non-neutralizing antibody effector functions can also potentiate disease through the delivery of infectious particles to

macrophages via immune complex uptake through Fc-receptors, as has been shown for antibody dependent enhancement (ADE) in the setting of dengue virus infection. Thus antibody Fc-effector functions represent a double-edged sword that are critical for viral clearance but can also be hijacked by viruses to enhance their capacity to gain entry into the host. As we gain a deeper appreciation into the antibody Fc-functions that provide the greatest levels of protection, future vaccination strategies may be rationally designed to selectively drive protective functional antibody profiles that avoid any antibody-enhancing effects.

Summary

The innate immune system composed of physical barriers, commensals, effector proteins, and an array of distinct immune cells collectively act with near instantaneous speed to protect against infection as well as set the stage for adaptive immune responses. The adaptive immune system, composed of T- and B-cell responses, represents an evolutionary marvel that permits the generation of nearly an infinite number of possible perpetually evolving, antigen-specific adaptive immune cells able to recognize, control, clear, and destroy any foreign antigen the immune system may encounter. However, the induction of this network of antigen-specific cells is heavily influenced by the innate immune system that clearly qualitatively shapes the functional activity of this arm of the immune system. Thus, the two arms of the immune system, the innate and adaptive arms, are intimately intertwined to ensure the evolution of the most effective immune response to ensure both the survival of the host following primary infection but also long-lived memory to rapidly clear and eliminate the pathogen upon reinfection.

Conclusion

Over the course of human evolution, we have developed elaborate mechanisms to defend ourselves from the persistent threat of viruses. Although many of the primordial mechanisms evolved in humans still persist today as components of our innate immune response to viruses, we have developed more elaborate means to protect ourselves through the evolution of the adaptive immunity. Thus the collaboration of the innate and adaptive arms of the immune system provides life-long immunity against these opportunistic pathogens.

Immunity to viruses is still incompletely understood and new cellular responses, innate sensing, and host restriction factors are identified every year. However, several layers of the antiviral immune response have been identified and briefly summarized here:

1. The most superficial part of the antiviral defense system is a layer of commensal microbacteria followed by physical barriers that include keratinocytes of the skin, mucus on mucosal surfaces, and several layers of mucosal membranes that can sense invading pathogens through PRRs. These layers are categorized as **barrier immunity**.
2. Intertwined with the barrier immunity is the **innate immunity** that senses invading pathogens based on PAMPs that are unique to microbes. Several PRRs have been discovered, including TLRs, NLRs, RIG-Is, and CLRs, and it is likely that there are several more still undiscovered. The sensing of the PAMPs happens either extracellularly or intracellularly in phagocytes (mDCs, pDCs, neutrophils, macrophages, etc.). These cells respond to the respective PAMPs accordingly and attract further cells of the innate and adaptive immune system or trigger the upregulation of host restriction factors such as TRIM5α, APOBEC, or tetherin.
3. A subset of the innate immune response, the NK cells, can also recognize specific antigens of invading viruses and are on the border of the adaptive immune response, exhibiting memory.
4. The innate immune response shapes **adaptive immunity** that consists of CD4 T and CD8 T cells as well as B cells. The development of adaptive immunity is complex but the resulting immune responses are highly specific for the invading pathogen and can form memory that will protect against a secondary re-encounter of the pathogen.
5. CD8 T cells are the main cell subset involved in killing intracellular pathogens such as viruses, but CD4 T cells can also acquire this ability as well. However, CD4 T cells are largely involved in generating a host of different functions required for the overall orchestration of the immune response to any infection.
6. Simultaneously with the development of CD4 and CD8 T cells, virus-specific B-cell responses develop aimed at generating copious amounts of antibodies that can attack the incoming pathogen. The maturation and characteristics of these antibodies are dictated in the GC by Tfh cells, aimed at generating both high-affinity as well as functionally relevant antibodies.
7. Antibodies can have many different functions. Besides neutralizing the agent, they can also recruit cells of the innate and adaptive immune systems, via Fc-receptors, to drive rapid control and clearance of pathogens. The activity of antibodies is dictated by the constant region of the antibody and may be exploited by vaccination strategies.

Thus, with our evolving understanding of the immune response to viruses, the opportunities to rationally develop novel therapeutics and vaccine strategies to prevent infection and/or disease are endless. Thus biomedical research efforts must continue to dissect the remarkably rich complexity of our immune response to viral infections and to provide insights for the development of medical interventions to end morbidity and mortality caused by these pathogens.

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Immunization Against Viral Diseases

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Vaccines against viral infections are perhaps the greatest of all biomedical achievements for preventing disease and improving the public health. The most notable example is the success of smallpox vaccination. The practice of variolization (mechanical attenuation and intentional low-dose infection) to reduce the virulence of subsequent smallpox infection was started more than 1,000 years ago in India and China. However, smallpox vaccination was first performed by Jenner in 1796. This event marked the beginning of modern vaccine development and the first clinical test of vaccine efficacy. An expansive, sustained world health effort with global cooperation and leadership in combination with creative approaches, like the ring vaccination campaign, resulted in the 1980 WHO declaration that naturally occurring smallpox had been eradicated. Numerous other vaccines have had a significant impact on the severity and frequency of viral diseases, and many previously common viral illnesses are rarely encountered in modern clinical care (Table 1). In particular, vaccines against hepatitis A and B, poliovirus, measles, mumps, varicella, and rubella have markedly reduced the frequency of these infections in developed countries. Unfortunately, many of these infections remain major health problems, particularly in communities who choose to opt out of standard childhood immunizations (1) and in the developing world. A description of viral vaccines available for use in the United States is shown in Table 2.

Effective immunization safely induces a vaccine-specific host immune response capable of either preventing infection or attenuating illness. Historically, viral vaccine immunogens were created by either inactivating or attenuating whole virus and were tested empirically with a rudimentary understanding of basic viral pathogenesis or protective immune responses. Newer generations of viral vaccines often involve a detailed understanding of the virology and immunology specific to the pathogen and utilize molecular biology approaches to produce vaccine antigens. This chapter will review current viral vaccine practices and concepts and indicate future directions for vaccine research and development.

GOALS OF VACCINATION

The goal of immunization against viruses is to improve both individual and public health by preventing or modifying virus-induced disease in a person and preventing or reducing the spread of infection in a population. When individuals do not participate in vaccination efforts against epidemic diseases, there are potential consequences for the person but greater consequences for public health (2). Vaccination is one of the few opportunities for a personal health benefit to be amplified as a benefit to the greater population.

Prevention of Infection or Disease

Active and Passive Immunization

Immunization is defined as induction of an antigen-specific host immune response by exposing the host to antigens representing, or comprised of those in, the wild-type pathogen. "Active immunization" to a virus can be induced by natural infection or by vaccination. The term implies an induction of immunological memory. "Passive immunization" refers to a transfer of temporary immunity to the host, which will not provide immunologic memory, but provides transient immune-mediated protection from infection or disease. The most common example of passive immunization is the transfer of disease-specific immunoglobulins into a host (3). In the cases of rabies and hepatitis B exposure, both passive (immunoglobulin) and active (vaccine) immunization are commonly used together to induce both rapid and long-lasting protection. Currently, there is only one monoclonal antibody (mAb) approved and in widespread use against an infectious disease. Palivizumab is used to prevent severe disease from respiratory syncytial virus (RSV) in premature infants. There are mAbs targeting HIV, West Nile virus, Ebola virus, and other infections in development and are likely to be used in future clinical practice for therapy or prevention of specific viral infections.

Prophylactic Vaccination

Most vaccines are designed to prevent virus infection in the individual. In most circumstances vaccine-induced immunity does not completely block infection but prevents disease by aborting or rapidly clearing infection through blocking transmission of virus to the target organ or preventing the indirect consequences of virus infections. Incomplete protection from infection has no clinical consequences for viruses that are typically self-limited and do not persist. For example, the inactivated polio (Salk) vaccine prevents dissemination of the virus to the anterior horn cells but does not prevent replication and shedding of poliovirus in the gut. The licensed hepatitis B virus (HBV) and human papillomavirus

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TABLE 1 Impact of licensed vaccines on annual prevalence of selected viral diseases reported in the United States

Viral disease	Peak year(s)	Peak prevalence	Modern prevalence	Percent reduction
Hepatitis A	1971	59,606	1,781[a,b]	97
Hepatitis B	1985	26,654	3,050[a,b]	88.6
Measles	1958–1962	503,282	187[a,b]	>99.9
Mumps	1967	185,691	584[a,b]	99.7
Polio	1951–1954	16,316	1[a,b]	>99.9
Rubella	1966–1968	47,745	9[a,b]	>99.9
Congenital rubella	1966–1968	823	1[a,b]	99.9
Smallpox	1900–1904	48,164	0	100

[a]http://www.cdc.gov/mmwr/preview/mmwrhtml/mm6253a1.htm
[b]2013 data.

(HPV) vaccines have a major goal of preventing a delayed clinical consequence of viral infection, liver cancer, or cervical, vulvar, vaginal, and anal cancers, respectively (4, 5). For viruses like HSV, HCV, or HIV that can become persistent or latent, vaccine-induced immunity to control or prevent virus infection needs to be more stringent and be achieved as soon after virus exposure as possible to prevent or minimize the viral reservoir. This is one of the great challenges in developing vaccines against these types of viruses.

Postexposure Vaccination

In some cases, vaccines can be effective even when administered after exposure to the pathogen, as for rabies and hepatitis B. Notably, individuals can be protected against a fatal outcome if vaccinia virus vaccine is administered up to four days after variola exposure, despite the relatively rapid progression of smallpox. For postexposure immunization to work, the vaccine must be highly immunogenic, and success is more likely when the pathogenesis involves a relatively long incubation period between infection and disease onset.

Vaccination to Control Viral Reactivation

Some vaccines can also be used therapeutically after long-term virus infection has become established in the host. This could be done to bolster immunity and prevent reactivation of latent infection or to induce additional immune effector functions in order to control persistent infection. The only licensed vaccine for postinfection prophylaxis is targeted against herpes zoster reactivation that may happen in persons with prior varicella infection as immunity wanes. Immunization of elderly adults with live-attenuated varicella-zoster virus (VZV) vaccine during latency reduces disease burden, the incidence of herpes zoster, and postherpetic neuralgia (6, 7). Diseases such as HSV-1, HSV-2, HCV, or HPV, which produce latent or persistent infection with periodic clinical flares or long-term clinical consequences, are candidates for therapeutic vaccine development, although achieving new responses to clear persistent virus in the setting of preexisting immunity is still an unproven concept. HIV-1 is also a target for therapeutic vaccine development, but the task will be more difficult for viruses like HIV that cause immunological impairment as a direct result of infection. Even for viruses like human papilloma virus (HPV), for which there is an effective prophylactic vaccine, the efficacy of therapeutic vaccination to either clear persistent virus or to treat cervical cancer will be difficult to achieve and will require a fundamentally different approach.

Herd Immunity

As an increasingly large fraction of a population is immunized against a particular pathogen, an indirect beneficial effect occurs that is greater than anticipated relative to vaccination rates. "Herd immunity" occurs as both transmitters of virus and susceptible persons are reduced in the population, and fewer incident cases of infection occur in the unprotected portion of the population. This results in a greater than expected decrease in the prevalence of infection and also means that if only a few individuals are not immunized, they will benefit from the immunity of those around them. This is an important consideration in the protection of individuals who have impaired immune systems and may not have the capacity to respond to vaccination. Rubella immunization is a practical example of herd immunity. The major goal is to prevent fetal abnormalities caused by intrauterine infection. Therefore, both males and females are immunized in the United States even though immunization has little direct benefit to males other than prevention of a relatively mild illness. Another type of herd immunity can occur when persons receiving live virus vaccines (e.g., oral poliovirus vaccine) transmit the attenuated vaccine strain to other susceptible contacts (8, 9). While this type of spread can improve vaccine coverage, depending on the stability of the attenuating mutations, if vaccine virus reverts to wild-type it could also have untoward consequences. Understanding the concept of the basic reproductive number (R_0) of a pathogen in a population helps to explain the concept of herd immunity. R_0 is the number of new infections that occur through transmission from a single individual. If R_0 is >1, then epidemics will expand, and if R_0 is <1, epidemics will contract. If vaccinees are protected from infection or if infected vaccinees have a shorter period of shedding, the number of transmission events from each infected person will drop. A rule of thumb to estimate the percentage of protected individuals in a population needed to achieve herd immunity is $(1—(1/R_0)) \times 100$.

Disease Eradication by Vaccination

The ultimate goal of vaccination is to achieve disease eradication. This is theoretically possible for viruses (like variola) that have no animal reservoir, do not cause persistent or latent infection, do not undergo major antigenic change, and exhibit distinctive clinical signs of disease to enable reliable recognition. Polio and measles meet these criteria, and stuttering progress has been made in ongoing efforts to eradicate these diseases through vaccination. Beyond those two, there are no viral diseases that are immediate candidates for eradication. With the ever increasing

TABLE 2 Vaccines licensed in the United States for use against viral diseases

Vaccine	Type	Route	Cell substrate	Trade name/sponsor	Comments[b]
Adenovirus	Live	Oral	Human diploid fibroblasts	Barr Labs Inc	Types 4 and 7 approved for military personnel 17 through 50 years of age and recommended by DoD for military recruits entering basic training
Hepatitis A	Inactivated	IM	Human diploid fibroblasts	Havrix/GSK	
				VAQTA/Merck	
				Twinrix (Hep A & B combination)/GSK	
Hepatitis B	Recombinant subunit	IM	Yeast	Recombivax HB/Merck	
				Energix-B/GSK	Booster: in specific circumstances (see ACIP guidelines)
	virus-like particles			Twinrix (Hep A & B combination)/GSK	
Human papillomavirus	Recombinant, virus-like particles	IM	Yeast	Gardasil/Merck	Types 6, 11, 16, 18
			baculovirus	Gardasil 9/Merck	Types 6, 11, 16, 18, 31, 33, 45, 52, and 58
				Cervarix/GSK	Types 16 and 18
Influenza A H1N1 2009 Monovalent Vaccine	Inactivated	IM	Embryonated chicken eggs	No trade name/CSL Limited	
				No trade name/ MedImmune LLC	
				No trade name/ID Biomedical Corporation of Quebec	
				No trade name/Novartis Vaccines and Diagnostics Limited	
				No trade name/Sanofi Pasteur, Inc.	
Influenza A and B	Inactivated[a] or live	IM or intranasal	Embryonated hen eggs	FluLaval/ID Biomedical Corp of Quebec	Specific H3N2, H1N1, and B strains selected annually; trivalent and quadrivalent compounds
				Fluarix/GSK	
				Fluvirin, Agriflu, Flucelvax/ Novartis	
				Fluzone/Sanofi	
				Flumist (live)/MedImmune Vaccines	
			Insect cells	Flublok/Protein Sciences Corporation	
Influenza H5N1	Inactivated	IM	Embryonated hen eggs	No trade name/Sanofi	Based on the Vietnam Strain of H5N1; for National Stockpile only
Japanese encephalitis	Inactivated	SQ	Mouse brain	JE-Vax/Research Foundation for Microbial Diseases of Osaka University	Booster: one year or more from previous dose
			vero cell	Ixiaro/Intercell Biomedical	
Measles	Live	SQ	Chicken embryo fibroblasts	Attenuvax/Merck	
				M-M-R II (measles, mumps, rubella combination)/ Merck	
				Proquad (measles, mumps, rubella, varicella combination)/Merck	

(*Continued*)

TABLE 2 Vaccines licensed in the United States for use against viral diseases (*Continued*)

Vaccine	Type	Route	Cell substrate	Trade name/sponsor	Comments[b]
Mumps	Live	SQ	Embryonated hen eggs and chicken embryo fibroblasts	M-M-R II (measles, mumps, rubella combination)/ Merck	
				Proquad (measles, mumps, rubella, varicella combination)/Merck	
Poliovirus (IPV)	Inactivated	SQ	Vero monkey kidney cells	IPOL/Sanofi	Serotypes 1, 2, and 3
					Booster: offered for travel to at-risk areas
Poliovirus (OPV)	Live	Oral	Monkey kidney cells	N/A	No longer distributed in the US
Rabies	Inactivated	IM or ID	Human diploid fibroblasts	Imovax/Sanofi	2 formulations in US. Purified chick embryo cell culture (PCEC) formulations are contraindicated in egg allergy patients
			Chicken fibroblasts	RabAvert/Norvartis/GSK	
					Booster: recommended for at-risk populations
Rotavirus	Live	Oral	Vero monkey kidney cells	RotaTeq/Merck ROTARIX/GSK	Bovine, pentavalent vaccine attenuated human virus vaccine
Rubella	Live	SQ	Human diploid fibroblasts	M-M-R II (measles, mumps, rubella combination)/ Merck	
				Proquad (measles, mumps, rubella, varicella combination)/Merck	
Smallpox, live	Live	ID scarification	Vero monkey kidney cells	ACAM2000/Sanofi	
Varicella	Live	SQ	Human diploid fibroblasts	Varivax/Merck	
Yellow fever	Live	SQ	Embryonated hen eggs	YF-Vax/Sanofi	Booster: 10 years
Herpes zoster	Live	SQ	Human diploid fibroblasts	Zostavax/Merck	

GSK = Glaxo SmithKline Biologicals; Merck = Merck & Co.; Novartis = Novartis Vaccines and Diagnostics Ltd; Sanofi = Sanofi Pasteur, SA; IM = intramuscular; ID = intradermal; SQ = subcutaneous.

[a]Inactivated influenza vaccine is administered intramuscularly.

[b]Durability and Booster information sourced from CDC.

capability in genome sequencing and molecular biology, even if a virus can be eradicated from nature, there will be full-length genome sequences (e.g., polio) and laboratory reservoirs (e.g., variola) that will remain.

IMMUNOLOGIC BASIS OF VACCINATION AGAINST VIRAL DISEASES

Innate immune responses are an important component of the immune system and provide an early, rapid-onset defense against pathogens (see Chapter 16). These responses do not require previous exposure, are generally not antigen specific, and are not thought to confer immunologic memory. The innate immune system is universal, and all cells possess some inherent capacity for protection. In contrast, adaptive immunity is antigen specific and requires an activation and amplification phase as it responds to a primary antigen exposure. This is accomplished primarily through specialized B and T lymphocytes and is characterized by immunologic memory with the capacity for rapid, specific effector responses upon reexposure to a previously recognized antigen. Many aspects of innate immunity intersect with elements of the adaptive immune response by providing mechanisms for recognizing threats, strengthening the engagement and activation of adaptive responses, and even sharing effector mechanisms.

Antiviral immunity is mediated by both innate and adaptive immune responses. However, vaccine-induced immunity depends on immunological memory, which is an inherent property of adaptive immune responses. Vaccination establishes preexisting antibody and memory populations of T and B cells that change the next encounter of a host with a

particular virus. Pre-existing antibody can reduce the initial number of infected cells and pace of viral replication, resulting in an attenuated disease course. An anamnestic response refers to the rapid increase in serum antibody that occurs after infection in a preimmune individual, indicating the presence of B cells that can rapidly expand as plasmablasts to produce antibody. A higher precursor frequency of virus-specific T cells can also attenuate disease through subtle changes in the timing and magnitude of responses. Vaccine design, formulation, and delivery will influence the specificity, quality, and location of T-cell responses and change the clinical expression of a subsequent virus infection. While it is the effector mechanisms of the adaptive immune response that are responsible for directly mediating vaccine-induced antiviral immunity, innate immune mechanisms are relevant to the pattern and quality of virus-specific responses that are predestined during the time immediately after vaccination.

Innate Immunity

Pathogen Recognition

Janeway predicted that the immune system had mechanisms to recognize molecular patterns associated with pathogens and coined the term PAMPs (pathogen-associated molecular patterns) (10). That hypothesis has been validated through the discovery and description of toll-like receptors (TLRs) that recognize a variety of molecules typically associated with microbial pathogens (11) (Fig. 1). Some of these TLRs appear to have specifically evolved to recognize molecules within endosomes associated with viruses including double-stranded RNA (TLR-3) and single-stranded RNA (TLR-7 and TLR-8). Other pathogen recognition receptors (PRRs) have evolved to recognize dsRNA in the cytosol such as the RNA helicases, retinoic acid-inducible gene (RIG)-I, and melanoma differentiation-associated (MDA) gene 5, or protein kinase receptor (PKR) (12). TLRs have also been noted to detect viral proteins such as the TLR-4 recognition of the RSV F glycoprotein (13). Triggering TLRs and other PRRs leads to an integrated set of responses by antigen-presenting cells (APCs) through interferon regulatory factors (IRFs) and NFκB transcription factors that control the induction of genes involved in the antiviral response. The process of pathogen recognition is an important consideration when designing the adjuvant and delivery vehicles used in vaccine formulations (e.g., AS04 in Cervarix targeting HPV [14–16]). These activating stimuli prepare the immune system for recognizing specific antigenic sites on the virus that can be targeted by adaptive immune responses.

Antigen Presentation

The principle antigen-presenting cell for initiating adaptive immune responses are dendritic cells (DCs). The function of DCs varies as they progress through successive stages of maturation, the immature state generally being better for phagocytosis and the mature state better for antigen presentation. In addition, there are subpopulations of DCs that have distinct properties. In the future it may be possible to engage selected DC subpopulations and direct the patterns of immune activation. For example, plasmacytoid DCs (pDCs) express TLR9 and are a major source of Type I interferon (IFN) production. Activating pDCs during vaccination might be expected to induce strong CD8+ T-cell responses because of the known association of IFN-α and memory CD8+ T cells (17). It may be possible to augment pDC activation with CpGs, which are short palindromic sequences in bacterial DNA that serve as a ligand for TLR9. DCs usually first encounter antigen in tissue during an inflammatory event. After phagocytosis of the antigen or an apoptotic cell that contains antigen, they become mobile and traffic to regional lymph nodes. The mature DC in the lymph node presents processed antigen as peptides in the context of MHC class I or II molecules in combination with costimulatory molecules to activate epitope-specific CD8+ and CD4+ T cells, respectively. Therefore, DCs are important for the initial immunization event and are also

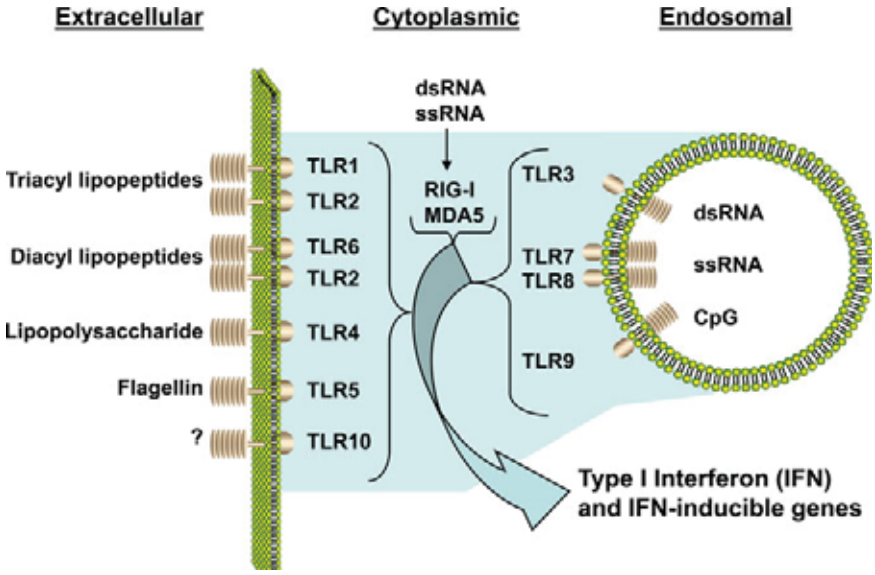

FIGURE 1 Recognition of viral pathogens. Eukaryotes have evolved mechanisms to detect potential microbial pathogens as they encounter the plasma membrane or penetrate the endosomal or cytoplasmic compartments of the cell. The molecules that interact with microbe-derived ligands trigger signaling pathways that lead to inflammatory responses. Harnessing the coordinated regulation of immune responses elicited by TLRs and helicases is a major focus of new adjuvant development for vaccines.

important after exposure to a viral pathogen to rapidly carry the viral antigen to lymph nodes to activate and expand vaccine-induced memory T cells in response to infection.

Immune Modulation

Immune modulation refers to altering the pattern of the immune response. This could involve the balance of response at various levels of specificity. For example, immune responses can be focused more on antibody or T-cell responses, CD4+ or CD8+ T cell responses, or Th1 or Th2 CD4+ T cell-responses. The patterns and characteristics of the immune response are often determined by the milieu of cytokines and chemokines and innate immune responses present at the time of initial antigen encounter (18). Therefore, vaccines are a powerful way to direct the pattern of subsequent immune responses to viral pathogens. For example, protein vaccines with alum adjuvants are more likely to induce T_H2 CD4+ T cells that produce IL-4, products with oil-in-water adjuvant are more likely to strengthen antigen uptake and activate CD4 T cells (19), and gene-based vector vaccines are more likely to induce Th1 CD4+ T cells and CD8+ T cells that produce IFN-γ. Similarly, combination adjuvant vaccines, i.e., TLR 7 adsorbed to alum, demonstrate immune activation with high antibody titers and Th1 induction (20–22).

Adaptive Immunity

Adaptive immunity involves the capacity for immunological memory and for evolving improved antigen recognition and effector functions after subsequent exposures to antigen. The major cellular components of the adaptive immune response include B and T lymphocytes. The relative importance of these two arms of the immune response varies with the particular viral infection. In general, antibodies are the major mediator of resistance to reinfection with virus, and CD8+ T cells are the major mediator of clearing virus-infected cells. CD4+ T cells comprise diverse phenotypic subpopulations. They can produce distinct cytokine and chemokines critical for the induction of robust antibody responses and important for "helping" CD8+ T cell responses; they also have potential for direct antiviral effector functions.

Antibody

Antibody is the key vaccine-induced effector mechanism for protecting against viral diseases. Antibodies are also a unique effector element of the adaptive immune response that can be maintained at protective levels prior to infection without causing harmful inflammation. The importance of antibody in protection against viral diseases has been demonstrated by the efficacy of passively administered antibody. Successful antibody prophylaxis has heralded subsequent vaccine development for polio, measles, varicella, hepatitis A, and hepatitis B. Passive antibody prophylaxis can also protect against some diseases for which no vaccine is available such as RSV and CMV, suggesting that active vaccination for these infections may also be possible.

Antibodies are generally thought to protect by "neutralizing" viral infectivity. Neutralization specifically refers to the property of reducing the number of infectious virus particles. Viruses are neutralized *in vitro* by antibodies using a wide variety of mechanisms. Antibodies can cause viral aggregation to effectively reduce the number of infectious units, inhibit virus attachment to cells, inhibit virus entry after attachment by inhibition of virus-cell fusion, or inhibit the release of newly formed virions from infected cells. Both quantitative and qualitative features of antibodies affect neutralization potency. These include concentration, antibody-to-virus ratio, valency, state of polymerization, affinity, avidity, isotype, ability to bind the polyimmunoglobulin receptor (pIgR), ability to fix complement, and specificity for a particular antigenic site (23). Additionally, antibodies may control infection by mechanisms such as antibody-dependent cell-mediated cytotoxicity (ADCC) where an infected cell displaying viral antigen on the surface binds the antigen-specific Fab region of the antibody. This allows the Fc region of the antibody to bind to Fc receptors of ADCC effector cells, classically CD16 on natural killer cells, resulting in death of the infected cell. Supporting this, *in vivo* studies of neutralizing antibody protection against simian-human immunodeficiency virus (SHIV) suggest that the presence of the Fc receptor is needed for optimal virus neutralization and may be more important than complement binding (24).

Ideally, vaccine-induced protection will be mediated by antibody that has the right functional properties, magnitude, and specificity to completely neutralize virus and provide "sterilizing" immunity. Antibody is especially effective when the target organ for disease is distinct from the initial site of infection. For example, in measles, polio, hepatitis, or viral encephalitis, where viremia is required to cause significant clinical disease, vanishingly small amounts of preexisting antibody can protect. However, it is rarely possible to elicit sufficient antibody through vaccination to fully prevent viral infection. As an example, respiratory virus infections are not fully prevented through sterilizing immunity. Typically, responses to natural infection or vaccination limit infection to the upper airway and diminish disease severity but do not fully prevent infection. Therefore, vaccines that can induce both antibodies and virus-specific CD8+ T cell (cytotoxic T cells) to rapidly eliminate residual virus-infected cells provide an extra measure of certainty that virus-mediated disease will be attenuated.

Specific antigen bound to the B cell receptor triggers the B cell to proliferate and differentiate into antibody-producing plasma cells. Initially, about 5 to 7 days after antigen encounter, plasmablasts can be transiently found in blood. Some plasma cells take up residence in bone marrow where they can survive and maintain virus-specific antibody in serum for years (25). B cells can also undergo differentiation to become long-lived memory B cells. While these memory cells do not secrete significant amounts of antibody, they reside in the germinal centers of lymph nodes and express surface immunoglobulin that allows them to quickly respond to a subsequent encounter with a specific antigen. These subsequent encounters are responsible for initiation of affinity maturation of the antibody response accomplished through the process of somatic hypermutation.

Immunization can influence antibody-mediated immunity in many ways. First, by increasing the repertoire and the frequency of immunoglobulin receptors on memory B cells capable of recognizing a particular antigen, the kinetics of antibody production on subsequent exposure will be more rapid. Second, somatic hypermutation and B-cell selection in lymph node germinal centers results in antibody that has evolved from the original germline sequences. Therefore, repeated virus infections or immunization can lead to affinity maturation of antibodies. Consequently, multidose vaccine regimens or vaccination prior to primary infection can improve the affinity, magnitude, and duration of antibody persistence. Third, immunization with a vaccine formulation that promotes IFN-γ production will increase the production

of IgG1 and IgG3 subclasses. Complement fixation is mediated by the CH2 region of the antibody Fc domain of IgM, IgG1, and IgG3, and complement binding can sometimes improve the neutralization potency of antibody. In contrast, IL-4 promotes IgE and IgG4 production, which are less desirable responses and are also associated with allergic reactions. Fourth, repeated immunization can improve the breadth of antibody responses to a particular virus by recruiting new responses to different antigenic regions. For example, there are five major antibody binding domains in influenza H3 hemagglutinin (HA) involved in virus neutralization, and repeated exposures to HA are generally needed to induce optimal antibody to all domains (26).

Some pathogens, particularly those with genetic variability, can misdirect the antibody response, including that to vaccines, as a mechanism of immune evasion. This has been characterized as "original antigenic sin" in the case of antibodies to the receptor-binding domain of influenza (HA) (27). B cells induced by past strains of influenza that recognize the rim of the sialic acid binding pocket can be preferentially expanded by new drifted strains because of their high precursor frequency, even though binding affinity and neutralizing capacity may be limited. This delays the induction of high-affinity antibodies that can neutralize the new strain and emphasizes the importance of the initial antigen priming event for the pattern and effectiveness of subsequent immunity. Immunodominance is another phenomenon that can prevent optimal antibody responses. There are examples of this in antibody responses to HIV env glycoprotein, gp160, associated with conformational states, where the most immunogenic sites are not neutralizing targets, and antibodies associated with broad neutralization are more difficult to achieve. Another example is influenza, in which antigenic sights in the head domain of HA are the most immunogenic but elicit antibodies that tend to be subtype specific. Responses to the HA head dominate responses to the HA stem domain, which has epitopes that are more conserved between subtypes and is associated with eliciting broadly reactive herterosubtypic antibodies (28–30). Therefore, identifying approaches that can induce more durable responses to subdominant stem epitopes may reveal strategies for developing a more "universal" influenza vaccine (31, 32).

CD8+ T Cells

T-cell responses (cell-mediated immunity) are essential components of a successful immune response for many pathogens, especially for viruses. T cells express T-cell receptors (TCR), which recognize specific peptide epitopes bound to major histocompatibility complex (MHC) proteins on the surface of antigen-presenting cells (APCs). The cytotoxic lymphocyte (CTL) TCR recognizes a short 8 to 10 amino-acid peptide derived from an endogenously produced viral protein in the context of the MHC class I β2-microglobulin heterodimer. Virtually all cells express MHC class I molecules and can be recognized by CTLs if they become infected by a virus. However, not all cells are equipped to effectively initiate a CTL response. For vaccine induction of virus-specific CD8+ T cells, the antigen containing the relevant epitopes must be present in the cytosolic compartment of a dendritic cell that can process the antigen, be liberated by proteolysis, and the peptide be transported into the endoplasmic reticulum where it can associate with the appropriate MHC class I molecule. Antigen presentation must then be accompanied by costimulatory signals that can activate T cells with relevant TCRs to proliferate and differentiate into effector and memory cells. The process is facilitated by the cytokine milieu produced by the local inflammatory process and the APCs and can be attenuated by the influence of CD4+ FoxP3+ regulatory T cells (Tregs).

The importance of CTLs in recovery from viral infection is witnessed by the frequency of severe viral infections associated with cellular immune deficiencies, and by the diverse strategies viruses use to escape CTL killing (33). Herpesviruses, poxviruses, and lentiviruses in particular have evolved mechanisms for interfering with antigen presentation or effector molecules required for CTL activity. The influence of CD8+ CTL memory on the outcome of a subsequent virus exposure can be subtle and may depend on the absence or presence of other components of the immune response. The impact of the CTL response is critically dependent on the timing of response and the efficiency and specificity of cytolytic activity. The immune response gains advantage in cases where virus replication and spread is slower or when the immune response is accelerated. More targeted, rapid killing of virus-infected cells and less bystander killing will reduce immunopathology and generally improve the clinical outcome.

No vaccine based primarily on induction of CTL has been licensed, and this is generally a secondary goal to antibody induction in vaccine development. Advances in polychromatic flow cytometry (34, 35) and other technologies such as CyTof (36, 37), Fluidigm (38), single-cell evaluation by functional studies (39), and sequencing (40) have made it possible to define functional subsets of T cells with more precision (41) and may provide the necessary tools for effectively targeting CTL induction in the future. Preclinical studies and early-phase clinical trials have demonstrated the potential for effectively inducing T-cell memory with effector functions sufficient for protection against viruses such as simian immunodeficiency virus (SIV), hepatitis C virus, and Ebola (42–45). In addition to memory phenotype, TCR specificity, and avidity, there are other CD8 T-cell properties that may make them more likely to contribute to effective immunity. For example, some CD103+ CD8 T cells, termed tissue-resident memory cells, remain in barrier tissues at mucosal surfaces and are poised to rapidly recognize and clear virus-infected cells at the site of initial inoculation (46). Also, high magnitude CD8 T-cell effectors can be maintained against some epitopes in a process termed "memory inflation" when induced by cytomegalovirus (CMV) vectors that can become latent and sustain intermittent antigen expression (47). This provides a pool of effector T cells that can rapidly respond to new infections and could be exploited for vaccinating against pathogens that require T cell-mediated immunity (48).

CD4+ T Cells

CD4+ T cells recognize peptide epitopes associated with the αβ heterodimeric MHC Class II molecules present on professional APCs. They have been categorized into an increasingly complex array of subpopulations based on which transcriptional regulator they express, the pattern of secreted cytokines, and other functional properties (49–52). These include not only traditional T-helper (T_H) cells but also cells involved in regulating inflammatory cytokine responses (T_H17 cells) and T-regulatory (Treg) cells that modulate the function of CD8 T cells. These subpopulations are important in molding the overall pattern of the subsequent immune response to viral infection and can be influenced by vaccine design and formulation. For example, T_H1 cells are important for creating an interferon-γ (IFN-γ)-rich

environment that promotes better cytolytic activity in CTL and switching to more potent subclasses of antibody. T_H2 cells produce IL-4, IL-5, IL-9, IL-10, and IL-13, which promote B-cell growth and differentiation but can also be associated with allergic inflammation. Another CD4 T-cell subpopulation critical for vaccine-induced antibody responses are follicular T-helper (TFH) cells. TFH are CCR5+ cells that produce IL-21 and express the transcription factor Bcl6. They are required for stimulating B-cell responses in lymph node germinal centers (53). Antigen specific T_H1 and T_H2 cells can be detected following vaccination (54, 55), as can antigen-specific T_{FH}. CD4 T cells can also provide direct antiviral activity *in vivo* (56–59), although the contribution is less than that of CD8 T cells, which is consistent with the limited distribution of MHC class II.

CD4+ T-cell epitopes are more numerous on viral proteins than CTL epitopes (60), and some responses can induce a protective immune response in the absence of B cells or CTLs (57). CD4+ T cells secrete IFN-γ, TNF-α, and other soluble factors with direct antiviral activity. Interestingly, the processes involved in establishing CD4+ T-cell memory are distinct from those involved in CD8+ T-cell memory responses (61). Antigen processing and presentation, unlike CD8+ T cells, occur through the endocytic pathway, so induction can be achieved by killed virus vaccines or even purified proteins and does not require live virus or gene delivery approaches for cytosolic processing. CD4+ T cells do not proliferate to the same extent as CD8+ T cells during primary infection, and those producing IFN-γ only do not survive as long-lived memory cells (62). In addition, inducing antigen-specific CD4+CD25+FoxP3+ Tregs may dampen other components of the adaptive immune response and diminish effector T-cell mechanism upon subsequent exposure to virus (63). The full impact of the complex role of regulatory T cells remains under investigation but the immune response to viral infection and vaccination is certainly affected by Treg activity (64–67). Therefore, the overall pattern and impact of CD4+ T cell induction is an important consideration in vaccine design.

Mucosal Immunity

Mucosal immunity refers to the local adaptive immune response at mucosal surfaces, including oral, upper and lower respiratory tract, gastrointestinal tract, and vaginal mucosae. The mucosal surface is the primary portal of entry for most viruses, and antigen-specific humoral and cellular immune responses can be found at those surfaces. Theoretically, when antigen-specific immunity can be induced at a mucosal site at risk for future virus exposure, in addition to induction of systemic responses, overall protection should be improved (68). The live-attenuated nasally administered seasonal influenza vaccine is based on this principle (69). Poliovirus vaccines provide an example of how mucosal immunity influences wild-type virus replication patterns. The inactivated (Salk) poliovirus vaccine given parenterally produces antibody responses that block transmission of poliovirus from the gut via the circulation to anterior horn cells. This vaccine is highly efficacious for preventing paralysis but does not prevent infection and replication in the intestinal mucosa. In contrast, the live-attenuated (Sabin) poliovirus vaccine protects against infection and prevents wild-type virus shedding. The importance of measurable evidence of mucosal immunity for vaccine-induced protection against other viruses is controversial.

Traditionally, the hallmark of mucosal immunity has been considered to be the presence of IgA. Dimeric IgA present in plasma or produced locally is trancytosed through mucosal epithelium and when associated with J chain protein binds the polymeric immunoglobulin receptor prior to secretion. IgA is thought to be particularly important for defense against pathogens limited to mucosal surfaces, and the primary immunodeficiency described as selective IgA deficiency may generally result in an increased risk of respiratory infections, diarrhea, and autoimmunity. However, IgA deficiency has not been associated with increased frequency or severity of specific viral infections. Therefore, the importance of specifically inducing IgA through vaccination is not known. IgG antibody present in the systemic circulation is also significantly involved in protection against mucosal pathogens if present at a protective titer. In general transudation of IgG occurs more easily in the lung than in the nasopharynx, and the intestinal tract is even more resistant. IgA can be present at high concentrations in mucosal fluid and is more resistant to proteolysis in that environment. The concept of a common mucosal immune system where mucosal immunity at one site confers protection at other mucosal sites has been suggested experimentally (70–72). However, in general, directly immunizing the mucosal surface at greatest risk of exposure will improve protection at that site. Although not well understood, this phenomenon may be related to observations that suggest some effector cells can remain in tissue for long periods of time at the site of a prior infection or antigen exposure (71, 73).

FORMULATION, ANTIGEN CONTENT, DELIVERY OF VIRAL VACCINES

Most licensed and investigational viral vaccines fall into one of seven categories: live-attenuated vaccines or chimeric viruses, inactivated vaccines, subunit vaccines, virus-like particles, replication-competent or replication-defective vectors, or nucleic acid vaccines (Table 3). The type of vaccine used for a particular viral pathogen, its formulation or adjuvant properties, antigen selection, schedule, and route of administration will all have profound effects on the types of immunological effectors induced. Therefore, vaccine design should be informed by an understanding of the pathogenesis and the optimal form of immunity to a particular viral pathogen.

Adjuvants

Adjuvants are used to improve the magnitude, composition, quality, and duration of vaccine-induced humoral and cellular immune responses to viral antigens. Adjuvants have typically been empirically derived from natural products with immunostimulating properties. For example, the classic Freund's adjuvant is a derivative of mycobacterial cell walls. Analysis of Freund's adjuvant, which is a mineral oil emulsion containing an undefined mixture of trehalose dymicolate, phospholipids, and lipopolysaccharides, led to more distilled products such as monophosphoryl lipid A (MPL) and MF59, oil-in-water emulsions. The active component in ISCOMS (immune stimulating complexes) derived from extracts of the soap bark tree (*Quillaja saponaria* Molina) was found in the 21st HPLC peak of the bark extract, hence the designation QS21.

The underlying mechanism of adjuvants has not been well understood. Traditional adjuvant concepts suggesting the importance of particulate antigen complexes, creation of antigen depots to prolong antigen persistence, or induction of nonspecific inflammation have given way to a more

TABLE 3 Platform technologies used to deliver viral vaccine antigens[a]

Vaccine type	Prototypes	Induction of protective antibody	Induction of CD8+ T cell responses	Potential for mucosal delivery
Live-attenuated virus	Sabin polio vaccine (OPV), Flumist influenza vaccine, rotavirus vaccines	+++	++	+++
Chimeric live virus[b]	Recombinant parainfluenza, measles, or vesicular stomatitis virus	+++	++	+++
Whole killed or inactivated virus	Salk polio vaccine (IPV) or conventional influenza vaccine	+++	+/−	+
Viral protein(s), virus-like particles	HBsAg—Recombivax HB, Energix-B, Twinrix, HPV—Gardasil	+++	+/−	+
Subunit proteins	FluBlok rHA influenza vaccine, VZV gE[b]	+++	−	+
Replication-defective vectors[b]	Recombinant poxvirus or adenovirus vectors	++	+++	+++
Nucleic acid[b]	DNA or mRNA	+	++	+

[a]This table focuses on antigen delivery approaches and does not attempt to include all the licensed (alum, AS03, AS04, MF59) or unlicensed (AS01b, ISCOMS, TLR agonists) adjuvants that could be used to modify the formulations and properties of the vaccines.
[b]Investigational status in humans although licensed veterinary vaccines utilize some of these platform technologies.

precise understanding of how the innate and adaptive immune responses are linked. The discovery of TLRs and their importance in pathogen recognition and immune activation has led to new options for vaccine formulations based on specific TLR engagement. TLR ligands trigger signaling pathways that change the functional state of antigen-presenting cells and thereby control the pattern and timing of cytokine production, mobility, and expression of costimulatory molecules. Selecting individual cytokines or costimulatory molecules as vaccine adjuvants underestimates the complexity of the milieu and importance of timing involved in nascent immune responses. Using TLR ligands as vaccine adjuvants may avoid the unanticipated problems that arise when using individual molecular adjuvants in isolation (74) and provides a more authentic stimulus for generating primary immune responses. Many empirically discovered adjuvants are now understood to be TLR ligands. For example, muramyl dipeptide derivatives and monophosphoryl lipid A stimulate TLR-2 and TLR-4, respectively. Imidazoquinoline compounds such as imiquimod and resiquimod have been found to be agonists for TLR-7 and TLR-8 that are designed to recognize single-stranded RNA. Palindromic CpG sequences found in bacterial DNA are recognized by TLR-9. Using TLR ligands as adjuvants (75) or conjugates (76) will be an important thrust of future vaccine development (22).

Antigen Targets

The antigenic content in the vaccine confers specificity to the immune responses. For antibody-mediated protection, the surface glycoproteins and capsid proteins of viruses are the primary antigens to target in a vaccine-induced immune response. These are the proteins associated with tissue tropism, attachment, and entry. Most neutralizing antibodies recognize conformational epitopes, so generally it is important to mimic the native structure as much as possible. When possible, displaying antigens as virus-like particles or in ordered arrays will tend to improve antibody responses (77). In some cases (e.g., HIV-1) induction of broadly neutralizing antibodies to viral glycoproteins is extremely challenging because of the need for oligomeric structures, conformational evasion, variable glycosylation patterns, genetic variation, and highly antigenic regions that are functionally unimportant but divert the recognition of functionally important epitopes (78–80). There is usually more than one antigenic site associated with neutralization on a virus attachment protein, and to reduce the risk of immune evasion, antibodies should be elicited to multiple sites. Recent advances in human monoclonal antibody identification and structural characterization of viral surface glycoproteins and viral particles have significantly improved the options for atomic-level design of vaccine antigens for the elicitation of neutralizing antibodies (81). The best example of this comes from the fusion glycoprotein (F) of respiratory syncytial virus (RSV). Solving the crystal structure of neutralizing epitopes on the prefusion and postfusion conformations of trimeric F (82) guided the molecular stabilization of the pre-F conformation that displays multiple neutralization-sensitive epitopes not present on the post-F surfaces (83). In addition, the structural information provided the basis for designing a single scaffolded epitope that was able to elicit RSV-specific neutralizing activity (84), establishing a proof-of-principle for structure-based vaccine design.

Antigen selection for inducing CD8+ T-cell responses involves a different set of considerations. Since CTLs recognize processed peptide epitopes in MHC class I molecules, all viral proteins (surface proteins, internal structural proteins, and regulatory proteins) can serve as antigens because they are all produced in the cytoplasm and are susceptible to proteasomal degradation. Including a large portion of the viral antigenic content allows the APC to select the appropriate epitopes for association with the host MHC class I. Theoretically, another approach would be to build designer proteins with a high density of CTL epitopes separated by flanking sequences susceptible to processing. There are also theoretical advantages to using antigens that are produced in high abundance and early in the virus replication cycle. This might allow earlier recognition of the virus-infected cell and more rapid clearance. In addition, there may also be advantages to using internal viral proteins because they are typically more conserved in viruses capable of genetic variation. The principles for developing vaccines based on CTL-mediated protection are not well established at this time, and no vaccine has ever been licensed based purely on its ability to induce a T-cell response.

Vaccine Delivery

The location and durability of vaccine-induced immune responses are affected by route and schedule of vaccine delivery. Currently licensed live-virus vaccines are given on mucosal surfaces orally (rotavirus and polio), by intranasal spray (influenza), by needle and syringe (measles-mumps-rubella and varicella), or bifurcated needle in the case of vaccinia. Delivery of vaccines by needle and syringe intradermally (ID) may provide dose sparing (85–87). This finding has been demonstrated with the licensed hepatitis B vaccine. Large-scale safety and immunogenicity studies were conducted by the U.S. Army to evaluate the dose-sparing potential of intradermal delivery for the licensed HBV vaccine. ID delivery of the HBV vaccine was safe and induced humoral immunogenicity at a lower magnitude (approximately 50% reduction) and a slightly lower frequency of immune response than traditional intramuscular (IM) delivery (88). As new vaccine technologies emerge, new delivery approaches may include microneedles (89) or for DNA vaccines, needle-free injection systems such as Biojector® or Powderject®, or electroporation devices that have been reported to improve antigen expression and immunogenicity (90, 91).

Delivery vehicles can provide mechanisms to codeliver mixtures of antigens and adjuvant, protect antigens from a harsh environment, control the timing of antigen release, or carry antigens into subcellular compartments. A number of carriers for delivery of vaccine antigens are in development. For example, virus-derived proteins inserted into liposomal membranes are referred to as virosomes. Virosomes containing the hemagglutinin of influenza are used in licensed products (outside the United States) to deliver vaccine antigens for influenza and hepatitis A (41). ISCOMs are 40 nm cage-like structures composed of lipids and saponins that can deliver viral antigens to the cytoplasmic compartment for processing and CTL activation (92). Microparticles or nanoparticles produced from synthetic polymers can be formulated with viral antigens or gene-based vectors for controlled release or delivery to selected cells (93, 94). Conjugates with carrier proteins also allow access to the cytoplasmic compartment of the APC.

Schedule of Immunization

The schedule of vaccine delivery and concept of boosting is critical for establishing durable immunologic memory for many vaccines. In general, inactivated vaccines require at least two doses in a naïve host to establish a significant immune response. Even live viral vaccines benefit from booster doses (e.g., measles booster in adolescents). Boosting prolongs the duration of the memory immune response and it may alter the character or breadth of the response by expanding the repertoire of antigens that stimulate an immune response (54,95–98). The classic indication of memory induction of the humoral immune response is isotype switching from antigen-specific IgM to IgG production, detected in the serum. Antigen-specific memory T cells may be present even in the absence of detectable antibody response as in the case of hepatitis B vaccine nonresponders (those with a negative hepatitis B titer following the last vaccination in the series) (99).

Homologous boosting with the same vaccine is used for most licensed vaccines (e.g., hepatitis A or B). Heterologous boosting or the concept of using one vaccine approach for priming and an alternative vaccine approach for boosting (prime-boost) may be important in the development of vector-based vaccines (100, 101) or for focusing the immune response on a particular antigenic site (102). Empirically it has been observed that as the interval between priming and boosting increases, the magnitude of the immune response improves (103). More recently, the importance of the length of time between the prime and boost has been clearly demonstrated (104–106). Considerations must be balanced between lengthening the vaccination schedule for potentially improved efficacy, and shortening the schedule for efficiency, compliance, and earlier protection. Some insight into the value of lengthening the prime-boost interval has been gained by understanding the basic biology of T-cell memory. After immune stimulation, lymphocytes evolve through an activation phase in which cells are highly functional but susceptible to apoptosis from excessive stimulation. As the acute phase of the response resolves, a population of memory lymphocytes is established with the capacity for rapid expansion rather than apoptosis after antigen stimulation (107). As the tools for immune assessment improve, the rules for prime-boost interval may be better tailored for each vaccine modality and be supported by a stronger scientific basis.

LICENSED VACCINES FOR VIRAL DISEASES

There are now licensed vaccines for preventing disease caused by 15 viral pathogens and over 27 viral subtypes in the United States (Table 2). They can be divided by live viral vaccines and nonreplicating viral vaccines. The following sections discuss their general properties. The reader should also consult the pathogen-specific chapters for details on their use.

Live Viral Vaccines

Viral vaccines that contain live or replication-competent forms of the targeted viral agent are referred to as "live virus vaccines." In live virus vaccines, the infectious virus is attenuated genetically, by host range characteristics, or by route of delivery to reduce virulence. Attenuated viruses are sometimes derived from naturally occurring isolates found to have low virulence or altered tropism (e.g., poliovirus 2). In most cases they are generated by performing serial passages of the virus in cell lines from an unnatural host. The underlying mechanism responsible for attenuating a pathogen during serial passage is due to spontaneous mutations that are selected by adaptation to the conditions of repeated passage in embryonated eggs or cell culture. Some of these mutations are associated with diminished pathogenicity in humans. Another source of live attenuated vaccine viruses comes from the "Jennerian" approach. Related viruses that have another species as a natural host are often highly attenuated in human hosts (e.g., rhesus or bovine rotavirus vaccines). In the case of adenovirus, the wild-type virus with natural tropism for the respiratory tract is delivered through the gastrointestinal tract, thereby attenuating virulence by avoiding the preferred tropism.

Live virus vaccines induce immune responses that closely mimic those of the wild-type virus, including the display of surface glycoproteins in their native conformation, the primary target of neutralizing antibodies. Live viral vaccines typically activate all components of the immune response. Both antibody responses and virus-specific T-cell responses (including CD8+ T-cell responses) are induced to the potentially protective antigens contained in the virus. The entire antigenic content of the pathogen is delivered by the live virus vaccine approach, and the antigen load is ampli-

fied by replication. Another advantage of live virus vaccines is that they can often be delivered to susceptible mucosal surfaces and elicit mucosal immune responses. Live viral vaccines are also associated with more durable immune responses and generally require fewer and less frequent booster vaccinations (108, 109). While there may be immunologic and protective advantages to the live virus vaccine approach, live vaccines may also have increased potential for adverse events. In rare cases, live vaccines may induce a mild or attenuated form of disease. Live virus vaccines are generally contraindicated in immunocompromised patients and pregnant women or in patients in close contact with immunocompromised individuals due to the risk of viral shedding and transmission in the postvaccination period (110). While many live virus vaccines are generally easy to manufacture, the production in mammalian cell substrates could introduce unexpected contaminants, virulence properties can change, and they are more susceptible to adverse storage conditions.

Nonreplicating Viral Vaccines

Many killed or inactivated and subunit viral vaccines have also been licensed for the prevention of viral diseases (Table 2). These vaccines generally require at least two doses to invoke the optimal immune response and are generally safe and well tolerated. The major advantage of properly inactivated vaccines is that there is no risk of infection in the vaccinated patient or transmission of live virus to potentially immunocompromised close contacts of vaccinees. A disadvantage of inactivated or killed vaccines is that they typically require multiple booster injections in the primary series or later in life to maintain a protective immune response in the majority of individuals. Inactivated influenza vaccines grown in eggs have been used since the 1940s. Hepatitis A vaccine is an example of an inactivated virus vaccine where formulations are produced by infecting human diploid fibroblast cells lines (MRC-5) with hepatitis A virus strains that are attenuated in humans. Virus from the infected cell line is formalin-inactivated to produce a nonlive vaccine product (111). Hepatitis B vaccine represents a nonlive, subunit vaccine formulation. Subunit HBsAg vaccine is produced in yeast by recombinant DNA technology (112). The vaccine is noninfectious but can induce transient serum HBsAg positivity (113). The vaccine is given in a three-dose schedule to infants, adolescents, or adults and results in seroconversion in >90% of patients who receive the three-dose regimen (5). Vaccines for both HPV and hepatitis B viruses have also been licensed utilizing virus-like particle (VLP) technology (Engerix-B and Cervarix, GlaxoSmithKline; Recombivax-HB and Gardasil 9, Merck & Co.). VLPs mimic viral structure but lack genetic material needed to allow for replication, yielding noninfectious and efficient antigen presentation (114). A VLP-based vaccine for hepatitis E virus has been licensed in China (115–117) and additional research on VLPs for a broad array of viral targets including alphaviruses is ongoing (118).

Substrates and Additives in Vaccine Manufacturing

In the United States viral vaccines are regulated by the U.S. Food and Drug Administration Center for Biologics Evaluation and Research (CBER). Most viral vaccines are produced in cell substrates that involve mammalian, avian, insect, or bacterial cell culture. Good manufacturing practice (GMP) requires rigorous record keeping and validated standard operating procedures that minimize the risk of contaminants or adventitious agents (119). Vaccine preparations are produced in bulk and stored or formulated with diluents, adjuvants, and preservatives before packaging. Each step in the process in highly regulated biological products is complex and raises many theoretical issues that may require clinical consideration and must be balanced for each individual against the personal and public health benefits of vaccination. For example, the preservative thimerosal is an organomercurial compound used in trace or small amounts in only a few vaccine preparations (120). Thimerosal is metabolized to ethylmercury and has been controversial because of the neurotoxicity associated with methylmercury. Ethylmercury is more rapidly excreted than methylmercury, and thimerosal has been given to animals and humans at doses well above 1 mg/kg without evidence of toxicity. The highest ethylmercury content in a 0.5 ml dose of any currently licensed vaccine is approximately 25 μg, and extensive clinical studies have not shown a causal relationship between thimerosal and neurological events (121). However, because of ongoing unsubstantiated public perception that it may be associated with neurocognitive or neurotoxic events and diseases such as autism, and ongoing efforts to improve vaccine safety and confidence, thimerosal has been removed from most licensed viral vaccines, especially childhood vaccines (120).

CLINICAL MANAGEMENT OF VACCINATION

Vaccination Schedules

The childhood and adult viral vaccine dosing schedules are routinely updated and listed in complete form by the US Centers for Disease Control and Prevention at http://www.cdc.gov/vaccines/schedules/index.html and by the World Health Organization at http://www.who.int/topics/immunization/en/index.html.

When a vaccine regimen is given as a series of doses, the question may arise about what to do in the case of missed or delayed doses. In general, it is not recommended or immunologically necessary to repeat or restart a vaccination regimen or schedule. The vaccine schedule should be continued where it was left off, regardless of the duration of time since prior vaccination. If there is concern regarding compliance and immune response following the last dose of vaccine in a series, an antigen-specific antibody titer can be measured in patient serum to determine if the patient has developed an appropriate immune response. For example, in the case of the licensed hepatitis B vaccine, an anti-HBs antibody titer of ≥10 mIU/mL, determined by commercially available immunoassay, correlates with protection from infection.

Live and killed viral vaccines can be administered simultaneously as recommended in published vaccination schedules (http://www.cdc.gov/vaccines/schedules/index.html). The general recommendation for administering multiple live vaccines is to administer them on the same day, or if that is not possible, to separate live vaccines by at least four weeks to allow for an optimal immune response to each vaccine. The recommendation is based on the premise that in the acute period of immune response to a live vaccine, additional immune responses to other live vaccines could be diminished because of innate immunity. Combination vaccines, with multiple vaccines coformulated and therefore coadministered have become increasingly common, both for ease of administration and improved compliance. Only licensed combination vaccine products that have been

rigorously tested for safety, stability, and immunogenicity in combination with other vaccine(s) should be coadministered in a single syringe. Health care providers should follow manufacturers' guidance and label information and should not prepare off-label formulations or combinations of vaccines by combining separate vaccines into a single syringe at the time of vaccination. The formulations of different vaccine products may be incompatible or antagonistic and significantly alter the chemical and antigenic properties of the vaccine products. This type of off-label practice could potentially lead to adverse events or reduction of the vaccine-induced immune response.

It is important to review the recommended vaccine schedule on a routine basis. The recommendations change periodically in response to outbreaks of disease or newly available vaccine products. For example, after several years of fewer than 300 reported cases of mumps per year, there was a multistate outbreak in 2006 with 6,339 cases reported. These were primarily in college age students with a median age of 21 (122). The surge in U.S. mumps cases was preceded by a large increase in mumps in the United Kingdom in 2005. Many of the infected persons had been vaccinated, but it is likely that waning immunity, particularly in persons who had not received at least two doses of MMR vaccine, was in part responsible. Adolescents should receive a booster dose of MMR vaccine, especially if they plan to attend college or live in settings such as dormitories where crowding may exist. Vaccine-induced immunity is not as long lasting as immunity induced by natural infection, and as infection dynamics evolve, vaccination schedules and clinicians will need to adapt. For example, although the United States declared measles eliminated in 2000, there was a multistate outbreak in 2014 with 668 cases from 27 states reported and 178 cases reported in the first half of 2015 (123). The surge in measles cases in the United States has been linked to transmission among unvaccinated communities and was preceded by a large increase in measles in the Philippines in 2014.

An important consideration in the resurgence of these vaccine-preventable diseases has been the opting out of recommended vaccination schedules. Whether based on fears concerning autism links (124, 125), waning belief for the need for vaccines in the face of declining incidence rates, or general antivaccine sentiments, complacency and skepticism have driven diminished protection (126–128). Outbreaks of HiB, varicella, measles, and pertussis have resulted from the popularity of nonmedical exemptions for vaccines granted in many states (129–132). In response, and to protect infants, adolescents, and immunocompromised populations, some US states have enacted restrictions to personal belief exemptions in order to achieve immunization specifically against childhood diseases.

Contraindications to Vaccination

Prior to each vaccination the health care provider should assess the patient for possible contraindications to vaccination, including a history of adverse or allergic reactions to a previous vaccination, underlying illness, or allergy. For live vaccine administration the provider should assess the possibility of immunodeficiency or the potential for patient contact with an immunocompromised individual. Vaccination is contraindicated if there is a known hypersensitivity to the vaccine or to any of the vaccine components. Egg and gelatin allergies are important considerations because of the potential for reactions to vaccines that contain them and the prevalence of sensitivity of those components. Allergy consultation may be recommended if vaccination is necessary and there is concern about a potential vaccine or vaccine component allergy.

Vaccination of Immunocompromised Individuals

The issue of which viral vaccines should be administered to immunocompromised individuals is complex. Factors that affect the decision include the degree and type of immunosuppression, the overall state of health of the patient, and the type of vaccine to be administered. In general, the most effective way of protecting people with immunodeficiencies from vaccine-preventable diseases is to make sure the people around them are well vaccinated and are not transmitters of infection. If immunization is necessary, the focus should be on nonreplicating vaccines. Schedules for vaccinating selected subgroups of immunocompromised individuals can be found on the second page of the Recommended Adult Immunization Schedule Table at the CDC web site http://www.cdc.gov/vaccines by following the link to "immunization schedules."

Live viral vaccines are generally contraindicated in immunocompromised patients and in some cases if the patient has close contact with an immunocompromised person (i.e., smallpox vaccine). Patients undergoing chemotherapy or radiation treatment should avoid live viral vaccines, but in some cases (i.e., varicella) their close contacts should be vaccinated to protect the immunocompromised patient. Measles and varicella vaccine can be administered following cessation of chemotherapy (> 3 months or later for measles), if no other contraindication exists. Live viral vaccines, such as varicella or smallpox vaccine, are contraindicated in severely immunosuppressed individuals because there is a risk of disseminated infection with the vaccine virus that can be severe in these patients. Disorders associated with T-cell immunodeficiency are associated with severe disease more often than immunoglobulin disorders. An exception to this is severe enterovirus infection (including poliovirus) in persons with immunoglobulin deficiency.

Nonreplicating viral vaccines do not pose an increased risk to immunocompromised individuals, although the response to a vaccine may be diminished or absent depending on the ability of the adaptive immune system to recognize and respond to the vaccine antigens. For example, immune response to influenza vaccine has been shown to be reduced in some children with malignancy (133). Persons with renal failure on dialysis often do not make a sufficient antibody to hepatitis B vaccines that are otherwise extremely immunogenic (134). As noted, it is best to vaccinate when host responses are intact and to otherwise follow the national guidance for immunization of persons with selected immunodeficiencies.

In the case of pregnant women, guidelines suggest specific vaccinations before, during, and after pregnancy. Tetantus, diphtheria, and pertussis (TDaP) and influenza vaccination are approved and specifically suggested during pregnancy, and additional inactivated vaccines can be administered although discussion with health care providers is recommended (135).

Adverse Effects of Vaccination

Expected Adverse Events

Vaccines are an important public health measure and provide a safe and effective method to prevent infectious disease. Occasionally, vaccines have been linked to adverse events. Vaccine-related adverse events may be expected with systemic or local reactogenicity, as manifested by mild fever, myalgia, malaise, or vaccination site pain, tenderness,

and erythema. These reactogenicity symptoms are transient and in most cases, antibiotics, antiinflammatory agents, and topical medications are not required.

Allergic Reactions

In rare cases, unexpected adverse events may occur, including anaphylaxis resulting from an allergy to a vaccine component. Potential causes of vaccine-related anaphylaxis include reactions to residual egg or chicken protein in vaccines grown in chick embryo fibroblasts, or reactions to gelatin, which is a preservative in many vaccine formulations. Viral vaccines, which may contain trace amounts of egg or chicken protein and therefore are contraindicated in egg-allergic patients, include yellow fever vaccine and one formulation of the rabies vaccine. Although produced in a chick embryo fibroblast cell substrate, MMR vaccine is not contraindicated in egg-allergic patients due to a lack of egg or chicken residual proteins in the final vaccine product.

Measles, mumps, rubella, varicella, Japanese encephalitis, yellow fever, and smallpox vaccines contain gelatin as a preservative and are contraindicated in individuals with a history of gelatin allergy. Once a patient has had an allergic reaction to a vaccine, or combination of vaccines, further vaccination with those vaccines or with vaccines containing the same components should be avoided and only reconsidered after a careful allergy evaluation is performed. Consultation with an allergist is indicated if allergy to a vaccine or to vaccine components is suspected.

Severe Rare or Idiosyncratic Adverse Events Related to Vaccination

The National Childhood Vaccine Injury Act of 1986 and The Vaccine Injury Compensation Program (VICP) (established 1988) were established by the U.S. Congress as a "no fault" alternative to financially compensate patients or the families of patients who suffer from vaccine-related injuries. Viral vaccines covered by the VICP include hepatitis A, hepatitis B, HPV, seasonal influenza, measles, mumps, rubella, polio, rotavirus, and varicella. The VICP Vaccine Injury Table details the covered adverse events and time intervals from vaccination for each of the vaccines (136). Adverse events included in the table are anaphylaxis for most vaccines, encephalitis for MMR, chronic arthritis for rubella vaccine, and thrombocytopenic purpura for measles vaccine. While rare, if these adverse events occur within a specified time period after vaccination, and no other cause for the event can be identified, the patient may have a claim.

Prior to vaccination, health care providers should provide the patient or their guardian with a Centers for Disease Control and Prevention (CDC) Vaccine Information Statement (VIS) (137). This is required under U.S federal law for all vaccines covered by the VICP. It is strongly recommended for all other vaccines. The CDC routinely updates current VIS forms on the CDC website to be printed by providers and their staff. The provider should document the date and version of the VIS form and note that the VIS was provided in the medical record for each vaccination.

Severe vaccine-related adverse effects are uncommon, but the following section highlights several examples and the mechanisms implicated.

Smallpox

Routine smallpox vaccinations in the U.S. civilian population were discontinued in 1972 but were continued in the U.S. military until 1990. Due to concerns about the possible risks of bioterrorism attacks, the U.S. military and a portion of the U.S. civilian population began to receive smallpox vaccination again in the winter of 2002–2003. Although individuals at increased risk for predictable vaccine-related adverse events were excluded, an unexpected adverse event, myopericarditis, was seen in approximately 1 in 12,000 primary vaccinees (usually within 30 days) following Dryvax vaccination (138). This finding, along with previously documented risks of adverse events, has led to multiple contraindications for prophylactic smallpox vaccination, including past or present atopic dermatitis, compromised immune system (HIV/AIDS, autoimmune disease, most malignancies, immunosuppressive medications), known or possible coronary artery disease, cerebral vascular disease, pregnancy, and breastfeeding. In the event of a smallpox outbreak or exposure, these contraindications would likely be disregarded and public health officials would make revised recommendations at that time.

Respiratory Syncitial Virus (RSV)

A formalin-inactivated whole virus RSV vaccine (FI-RSV) was studied in infants and children in the 1960s, when Tween-ether and formalin-inactivated measles virus was still being used. The RSV vaccine did not prevent infection and was associated with enhanced disease that was particularly severe in the youngest age group <6 months (139, 140). Both FI-RSV enhanced illness and atypical measles, an aberrant illness caused by infection several years after vaccination with inactivated measles vaccine when neutralizing antibody activity had waned, are thought to be the consequence of a similar combination of immunological events. First, enhanced illness occurred when vaccine-induced antibody did not have significant neutralizing or fusion-inhibiting activity and did not prevent infection (141). Second, vaccine-induced antibody had complement-fixing properties, and immune complex deposition could be demonstrated in affected tissue (142). Third, there was an exaggerated CD4+ T-cell response associated with eosinophilia (139, 143). Animal models of FI-RSV and measles suggest this was caused by a Th2 (142) biased responses, with production of IL-4, IL-5, IL-13, and eosinophilia (144).

Rotavirus

An oral tetravalent rotavirus vaccine (Rotashield®) was licensed for use in the United States in 1998 but was withdrawn from use due to a possible link to intussusception (145). The vaccine was associated with an increased rate of intussusception of up to 1 per 2,500 vaccinees, primarily following the first dose of vaccine (146). Subsequent epidemiologic studies led to a significant decrease in the estimates and suggested that the early period of increase in intussusception seen after the first dose was compensated by lower rates after subsequent doses, resulting in no net overall increase in the number of cases in vaccinees less than one year of age (146). The cases occurred predominantly when the vaccine was started in children three months of age or older rather than in children who received the recommended schedule (two, four, and six months of age) (23, 147, 148).

Influenza

Adverse events (local and systemic reactogenicity) related to seasonal influenza vaccines are generally mild, including localized redness, swelling or mild fever, and muscle

aches. Intranasal live-attenuated influenza vaccine can also be associated with mild rhinitis-like symptoms, headache, muscle aches, and fever. There have been rare moderate to severe, unexpected adverse events associated with influenza vaccines. In 1976 the influenza vaccine (swine flu vaccine) was associated with Guillain-Barré syndrome (GBS) at a very low rate (approximately 1 to 10 per one million persons vaccinated). A causal relationship between influenza vaccine and GBS was not clearly identified, but due to the historical association it is recommended that people with a history of GBS speak to their physician before influenza vaccination.

Yellow Fever

Yellow fever virus (YFV) vaccine is generally considered one of the safest live virus vaccines and may be associated with mild reactogenicity in 25% of recipients. More serious conditions have been associated with YFV vaccine at very low frequencies. YFV vaccine-associated neurotropic disease has been reported in < 1 in 8,000,000 persons receiving the vaccine. This condition is generally transient and rarely fatal. A second severe, possibly fatal, but very rare adverse event associated with YFV vaccine is yellow fever vaccine-associated viscerotropic disease which is seen in one in 400,000 initial doses. This condition mimics fulminant yellow fever infection caused by wild-type yellow fever virus. This rare condition has been reported only following initial vaccinations and has not been reported to occur following booster vaccinations with YFV vaccine (149).

Reporting Vaccine-Related Adverse Events

Following licensure of a vaccine, adverse events that are clinically significant or unexpected as well as events listed in The Reportable Events Table (150) and in the manufacturers package insert should be reported to the Vaccine Adverse Event Reporting System (VAERS) (http://vaers.hhs.gov/). VAERS is a passive surveillance cooperative program between the CDC and the FDA. Any possibly related or temporally related vaccine side effects may be reported by any health care provider, patient, or patient guardian or representative. The VAERS system is public and represents unverified reports of possible vaccine-related adverse events. The VAERS system allows public health experts to detect rare adverse events that may not be discovered during vaccine clinical trials because of their low rates of occurrence (151). Potential vaccine-related concerns in the VAERS database are further investigated and/or verified by the CDC, Vaccine Safety Datalink Project (VSD) (152). Vaccine research and development would benefit from better-defined rates of unusual or severe conditions or illnesses in the general population so that occurrences that are temporally related to vaccine can be more clearly compared to the general population rate (unrelated to vaccine) (153).

LICENSED PRODUCTS FOR PASSIVE IMMUNIZATION

When active immunization is not possible, transient protection from disease or infection can be passively acquired by administration of human polyclonal immunoglobulin known to have a high titer of antibody against a specific viral pathogen or virus-specific humanized monoclonal antibodies. Like vaccines, licensed immunoglobulin products for use in humans are regulated by the U.S. Food and Drug Administration Center for Biologics Evaluation and Research (CBER). Human immunoglobulins are licensed for use in the United States against cytomegalovirus (CMV), respiratory syncytial virus (RSV), vaccinia, varicella zoster, rabies, and hepatitis B virus.

Preexposure Prophylaxis

In the past, prophylactic immunoglobulin was used for protecting travelers against hepatitis A but has been replaced by an effective vaccine. Currently, the only passive antibody prophylaxis in widespread use is for RSV. RSV immune globulin (RespiGam®) was indicated for the prevention of serious respiratory disease from RSV infection in high-risk children < 2 years of age but is no longer in use. A humanized monoclonal antibody against the F glycoprotein of RSV (palivizumab, trade name Synagis®) is indicated for the prevention of serious respiratory disease from RSV infection in high-risk children < 2 years of age. Risk factors for severe respiratory disease from RSV include bronchopulmonary dysplasia, premature birth (≤ 35 weeks), and hemodynamically significant congenital heart disease.

Postexposure Prophylaxis

Rabies immune globulin is given to nonimmune individuals in conjunction with active rabies vaccination following potential or confirmed rabies exposure, such as in the case of animal bites. The wound should be infiltrated with immune globulin at the time of or within eight days of the first dose of rabies vaccine. Hepatitis B immune globulin is administered to nonimmune individuals exposed to hepatitis B as postexposure prophylaxis and in that setting is usually given in association with hepatitis B vaccination. It should be administered within 24 hours of exposure to hepatitis B by needle stick, ocular, or mucosal exposure or within 14 days of a potential sexual exposure. Hepatitis B immune globulin is given to newborns of infected mothers (HBsAg positive) within 12 hours of birth and again at three months of age. Hepatitis B immune globulin is indicated only for prophylaxis and not for the treatment of hepatitis B (active or chronic). Vaccinia immune globulin is indicated for the treatment of smallpox vaccine-related complications, including eczema vaccinatum, progressive vaccinia, and severe generalized vaccinia. Varicella zoster immune globulin (VariZIG), approved in 2012 for the prevention of varicella infection in immunocompromised individuals exposed to varicella zoster virus and for those for whom varicella vaccine is contraindicated.

Adverse Effects and Contraindications to Passive Antibody

Human immunoglobulin products may contain trace amounts of human IgA. IgA deficiency is the most common cause of primary immunodeficiency diseases, affecting approximately one in 500 people, and is often undiagnosed. In rare cases, individuals with IgA deficiency can develop IgE against IgA and experience anaphylaxis upon exposure to human blood products containing IgA, including immune globulins. IgA deficiency is a relative contraindication to receipt of human immune globulin products and this should be considered prior to administration.

Immunoglobulin can inhibit the immune response induced by some live viral vaccines. This may occur because the presence of virus specific serum antibody binds to and inhibits live virus vaccine replication and therefore prevents

the intended immune response. Varicella vaccination can be inhibited up to five months following administration of human immune globulin containing antivaricella antibodies and rubella vaccination can be affected up to three months following immune globulin administration. Ideally, vaccination with varicella and rubella vaccines should be delayed by five and three months, respectively, following the administration of immune globulin. In cases where immune globulin is administered to a patient within three weeks after MMR or varicella vaccination, the immune response to those live vaccines may be blunted. In that situation, revaccination after three to five months is recommended if there are no other contraindications. Hepatitis B vaccine can be administered at the same time as hepatitis B immune globulin or ≥1 month following the administration of immune globulin. The immune response to yellow fever vaccine does not appear to be inhibited by the presence of immune globulin at the time of or following vaccination.

VACCINE DEVELOPMENT AND FUTURE DIRECTIONS

Vaccine development in the 21st century promises new paradigms for design, manufacturing, immune response evaluation, and timelines. While empiricism will always contribute to scientific advances, vaccine development is now more dependent on rational design and a deeper understanding of the underlying molecular pathogenesis of disease. One example of this is adjuvant development based on new discoveries and understanding of TLRs. Sequencing technology, advances in molecular virology, ease of isolating and characterizing human monoclonal antibodies, and improvements in the technologies and speed of solving antigen structures will support rapid identification of new pathogens and atomic level guidance for vaccine antigen design. In addition to the traditional platforms of live attenuated, whole virion-inactivated, and virus-like particle vaccines, there are new options for structure-guided vaccine antigen development, and a variety of gene-based delivery approaches using DNA, RNA, or microbial vectors that will become part of the repertoire of licensed vaccines. Vector-based and nucleic acid vaccines using gene delivery of vaccine antigens have already been approved for veterinary use for several viral diseases.

Structure-based vaccine development utilizes specific knowledge of how human mAbs interact with viral surface proteins and neutralize virus (82, 83, 154) and the process is iterative. Improved vaccine antigens can be used as probes to select B cells with specified properties for cloning antibody genes for improved monoclonal antibodies that can be used to better characterize vaccine antigens. In addition, an expanding number of technologies have allowed the evaluation T-cell responses at the single-cell level and has prompted the development and evaluation of T-cell based vaccine concepts in addition to the traditional concepts of immunity based on antibody responses. Even the way antibody function is evaluated has evolved from ELISA and basic neutralization assays to measurements of epitope-specific binding and functional assays (155), multiple approaches to assess Fc-mediated functions (156, 157), and the impact of glycoforms on antibody function (158). Rapid advances in human genetics have created the possibility of designer vaccines that can take advantage of certain host polymorphisms to improve immunity and avoid others to reduce side effects. New sequencing technologies will impact surveillance and virus discovery, better characterize genetic diversity and viral escape mechanisms, and the analysis of immune response ontogenies. They will also influence vaccine safety by application to the evaluation of biologics (159).

Significant biological hurdles still exist for developing effective vaccines to prevent viral infections with large disease burdens such as HIV-1, HCV, and HSV, as well as influenza where a more durable and broader "universal" influenza vaccine is needed. There is great effort to find effective vaccines for these viruses using a variety of new technologies to better understand pathogenesis and immunity and to design novel immunization approaches. For viral infections with the potential for sporadic epidemics or intentional release and high disease severity such as orthopox viruses, zoonotic influenza viruses, filoviruses, and flaviviruses, the challenges are both biological and strategic. The same is true for emerging pathogens with the uncertain potential for pandemic spread like Middle East respiratory syndrome (MERS) coronavirus. One approach for addressing the strategic issues involving emerging infections and biodefense has been to establish a stockpile of available vaccines and other prophylactic and therapeutic agents (160). To be effective, this concept will require a level of anticipation and significant political commitment to preparedness as illustrated by the West African Ebola crisis in 2014. Additional research and development is needed to establish platform vaccine technologies that can be rapidly adapted and deployed against new viral pathogens that will inevitably emerge. Innovative public and private partnerships will be needed to overcome the biological and logistical hurdles of vaccine development for emerging infections.

Solving the political and social obstacles that restrict the distribution and delivery of existing vaccines to regions of the world where they are not widely utilized would dramatically improve public health. GAVI, the Vaccine Alliance, has significantly improved global health by distributing new and underused vaccines to low- and middle-income countries (LMIC) and drives innovative vaccine-financing mechanisms. The vaccine manufacturing landscape is changing in part because of new technologies that promote more disposable and modular equipment for good manufacturing practice (GMP) and in part because of expanding global capacity for vaccine development. For example, Zhu and colleagues brought a hepatitis E vaccine to market following a large-scale efficacy study in a general population at risk of infection in China. The purified recombinant hepatitis E antigen vaccine, HEV 239, trademarked as Hecolin, received approval by China's SFDA in December 2011 and is manufactured by Xiamen Innovax Biotech Co. (116, 117). More recently, a vaccine targeting rotavirus developed and manufactured in India received licensure in 2014 and was subsequently introduced into the country's national immunization program. The live-attenuated monovalent human-bovine (116E) vaccine was assessed in a large scale efficacy study of over 6,799 Indian infants and proved efficacious and serves as an example of efficient viral vaccine development (161). In addition, Brazil is playing a large role in manufacturing and advanced evaluation of a dengue virus vaccine, providing another example of the changing paradigm. Even as manufacturing and distribution options improve in LMICs, additional investigation is needed to understand the biological basis for why vaccine immunogenicity is often diminished in developing country settings.

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Chronic Fatigue and Postinfective Fatigue Syndromes

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Chronic fatigue syndrome (CFS) is the label most commonly applied to a symptom complex characterized by unexplained, persistent, and disabling fatigue, when no clear implication of an infective or other cause for the prolonged illness can be inferred (1). Constitutional symptoms, including myalgia, arthralgia, sore throat, headache, and tender lymph nodes, are also common. In addition, complaints of unrefreshing sleep, irritability, and neurocognitive abnormalities, like short-term memory impairment and concentration difficulty, are typical (1). CFS is also commonly termed myalgic encephalomyelitis, particularly in the United Kingdom, based on a premise of inflammation within the central nervous system—although this remains unproven (2). The US Institute of Medicine recently proposed that the condition be renamed "systemic exertion intolerance disease" to reflect a phenomenon characteristic of the illness, which is an exacerbation of fatigue and other symptoms after exercise or activity, termed "postexertional malaise" (3). However, only the name CFS has been accepted in common use internationally.

Fatigue is a subjective and nonspecific symptom, present in at least 15 to 30% of patients in primary care settings (4, 5), where it is predominantly associated with minor psychiatric morbidity and transient infective illnesses (6). The relationships and boundaries between the ubiquitous symptom of fatigue in the community, the broadly inclusive symptom complex designated as CFS, and the more discrete entity of postinfective fatigue syndrome, are often blurred. Patients with postinfective fatigue represent a subset of those with CFS, although the size of this subgroup is uncertain as many acute infections are subclinical, are not investigated, and the causative agent for an apparent infective trigger for a fatigue syndrome is often not identified. In addition, the international case definition for CFS stipulates six months or more of disabling illness (1). Although many postinfective fatigue states are comparable in both symptom profile and functional impairment to CFS, the majority of episodes are of shorter duration (7).

Individuals suffering from acute infective illnesses typically develop a constellation of systemic symptoms, including feverishness, myalgia, arthralgia, and headache, as well as fatigue (8). In both humans and animals, infections are also accompanied by increased slow-wave sleep and stereotyped behavioral responses, including reduced motor activity, social withdrawal, and anorexia (8). These characteristic physical and behavioral correlates of infection result primarily from the host response to the pathogen, as they are reproduced in infections resulting from a wide range of microbiologic agents (9). In a minority of cases, selected symptom domains from the acute phase of the illness persist and cause protracted ill health, marked by fatigue and disability. Prospective evaluation of such symptoms following serologically documented Epstein-Barr virus (EBV) infection indicates that illness extending over several months, marked by fatigue, is common (Fig. 1) (10–13).

Over several decades, both CFS and postinfective fatigue syndrome have remained a focus of considerable research interest, including epidemiological studies, investigations of microbiologic, immunologic, neuroendocrine, metabolic, and psychologic hypotheses of etiology, and treatment interventions (reviewed in 14–18).

HISTORICAL PERSPECTIVE

A review of the history of the modern day phenomenon of CFS indicates that the disorder is unlikely to be new (19). Perhaps the earliest description comes from Sir Richard Manningham in 1750, who described "febricula," or little fever, as "listlessness with great lassitude and weariness all over the body . . ." (20). Other features included fleeting muscle and joint pains, as well as neurocognitive disturbance: "sometimes the patient is a little delirious and forgetful" (20).

The most widely known predecessor to the current label of CFS is the disorder termed "neurasthenia," which was first delineated by George Beard, an American neurologist, in 1869 (21). This disorder was best described by Cobb in 1920: "Neurasthenia is a condition of nervous exhaustion, characterized by undue fatigue on slightest exertion, both physical and mental, with which are associated symptoms of abnormal functioning, mainly referable to disorders of the vegetative nervous system. The chief complaints are headache, gastrointestinal disturbances, and subjective sensations of all kinds" (22).

Despite these vivid historical references, precise parallels cannot be drawn with any confidence between the current label of CFS (which requires systematic clinical and laboratory evaluations to exclude alternative diagnoses), to

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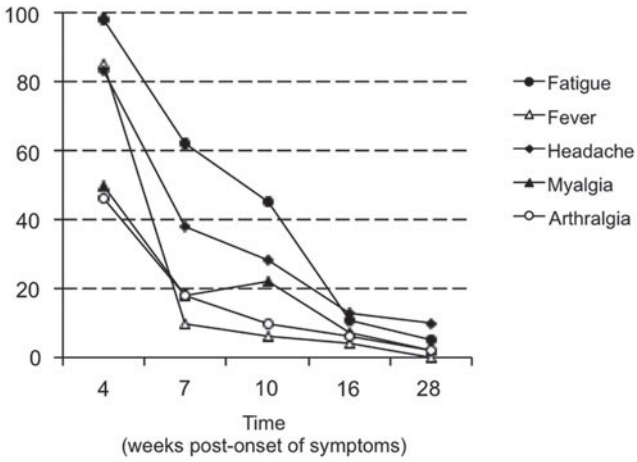

FIGURE 1 Time to resolution of symptoms in 50 patients monitored prospectively from the time of diagnosis of serologically documented acute EBV infection. The vertical axis indicates the percentage of the group reporting the symptom. (Used with permission by A.R. Lloyd, unpublished.)

TABLE 1 Infectious diseases putatively linked to fatigue syndromes[†]

Infection, categorized by type of pathogen implicated (1869–2015)		
Viruses	Bacteria	Parasites
Influenza	Typhoid fever	Malaria
St. Louis encephalitis	Streptococcal infection	Schistosomiasis
Epstein-Barr virus	Brucellosis	Toxoplasmosis
Hepatitis A	Lyme disease	Giardia
Yellow fever	Q fever	
Varicella	Leptospirosis	
Chikungunya		
Coxsackie B		
Cytomegalovirus		
Human herpes virus-6		
Ross River virus		
Mumps virus		
Retroviruses		
Human herpes virus-7		
Borna disease virus		
Parvovirus		
Dengue fever		
West Nile virus		

[†]Modified from reference (157).

disorders such as the *febricula* or *neurasthenia* of earlier centuries. The term neurasthenia, for instance, was used in a variety of different contexts, including as a synonym for general nervousness and evolving psychosis as the male equivalent of hysteria in women; as an alternative label for minor depression; and as a diagnosis of fatigue states in patients who were not apparently depressed (19).

Although even the first descriptions of neurasthenia included a link with febrile illness, the observation that specific infectious diseases precipitated a subsequent fatigue state came with the microbiologic revolution of the late 19th and early 20th centuries. Notable among the infections linked to postinfective fatigue were influenza (23), brucella (24), and EBV (25) (Table 1). This association of a largely subjective syndrome of chronic fatigue with discrete infections, like influenza and brucellosis, sparked considerable controversy. Studies conducted in the 1950s provided evidence that much of the continued morbidity was attributable to psychological factors. Retrospective studies following up patients after acute brucellosis failed to find bacteriological evidence of chronic infection (26). However, sensitive detection techniques, such as polymerase chain reaction (PCR), to identify low levels of persistent nucleic acids of the microorganisms, or immunoassays to identify microbial antigens, were not available. The critical issue of whether the fatiguing illness led to psychological disorder or, conversely, whether such disorder resulted from chronic ill health, was studied in a prospective evaluation of military personnel before the Asian influenza epidemic of 1957 (23). This study pointed to premorbid psychological vulnerability as an important determinant of protracted recovery after infection, although it should be noted that the number of cases available for this analysis was very small.

These controversies of microbiologic versus psychological determinants of postinfective fatigue have continued to the present. The first prospective evaluation of patients followed from mononucleosis illnesses suggested that both concepts are valid (10, 27, 28). In this study, 245 patients were enrolled with either infectious mononucleosis (77%) or upper respiratory tract infection (23%). Of these, 101 (41%) had a fatigue syndrome associated with significant functional impairment at the time of enrollment. The fatigue state persisted for one month or more in 71 individuals from this group (73%), for two months or more in 43 (43%), and for six months or more in nine (9%). The fatigue syndrome was most prevalent in those with mononucleosis documented to be due to EBV infection and was shown to be essentially independent of psychiatric diagnoses (10). By contrast, a well-controlled longitudinal study in general practice found that patients presenting with minor symptomatic infections, such as gastroenteritis, were not more likely to subsequently report chronic fatigue than patients presenting for other noninfective reasons (29). A prospective cohort study following individuals from the time of onset of serologically documented acute infection with three different pathogens identified a stereotyped postinfective fatigue syndrome (7). This study enrolled individuals after onset of infection with EBV, a herpesvirus with recognized latency; Ross River virus (RRV), a small, mosquito-borne RNA virus causing acute illness with arthritis; and *Coxiella burnetii*, the intracellular bacterium causing Q fever (7). The study documented that of 253 subjects enrolled, 29 (12%) experienced a prolonged illness characterized by fatigue, musculoskeletal pain, neurocognitive difficulties, and mood disturbance lasting six months or more and 28 (11%) met the diagnostic criteria for CFS after systematic assessment to exclude alternative medical and psychiatric explanations for the ongoing illness. The advent of a postinfective fatigue syndrome was predicted largely by the severity of the acute illness and not by demographic, psychological, or individual microbiologic factors. The fatigue state was present from the time of onset of symptoms of the acute infection and was stable in character over time, suggesting that the genesis of the postinfective fatigue syndrome is intrinsically linked to the host-pathogen interactions in the acute infection phase (7).

EPIDEMIOLOGY AND NATURAL HISTORY

CFS is a relatively common condition in the community. The disorder predominantly affects young adults with a peak age of onset of 20 to 40 years, often following a documented, or an apparent, infectious illness (30–33). CFS does not preferentially affect individuals from upper socioeconomic groups (contrary to the notion of "yuppie flu"), rather, studies in both adolescents and adults suggest that fatigue syndromes are more common in people from more socially disadvantaged groups (31, 34–36). Estimates of the prevalence of CFS in the general population (7, 30, 31, 33, 37) and studies in primary care settings (which record the cases only among those attending medical practice and are therefore subject to selection bias) suggest prevalence rates ranging between 0.04 to 2.6% (31, 34, 37, 38). These differences in prevalence result from differences in the method of ascertainment (e.g., self-report or physician report), and, in the case definitions used for the syndrome, notably in the extent of the assessments undertaken to exclude alternative medical and psychiatric diagnoses (39). Preliminary estimates of the incidence of CFS suggest annual rates of approximately 0.1 to 0.3% (33, 37, 40, 41).

The long-term outcome of patients with CFS has been evaluated predominantly in tertiary referral settings, where the patient populations are intrinsically biased toward chronicity and disability. In one such study, 65 of 103 patients (63%), who had long-standing symptoms and who had been enrolled in treatment trials, reported improvement in symptoms and functional capacity at follow-up approximately three years later (42). No alternative medical diagnoses became evident over this period. Complete recovery was uncommon (6%). By contrast, spontaneous recovery in cases of fatigue of shorter duration in primary care appears to be high (43–45). Prospective, community-based studies suggest that the prognosis for recovery is high when the illness is abrupt in onset (potentially indicating an infective trigger) and of short duration (6 to 12 months), but declines substantively when the illness has been present for several years (46–49). Similarly, the prognosis for recovery in postinfective fatigue syndrome is high, even after six months of illness have passed, especially in adolescents (7, 16). Even in those with long-standing illness, the natural history is predominantly of stable or slowly improving status, rather than deterioration (50, 51).

DIAGNOSTIC CRITERIA

The modern era of research in relation to CFS has been marked by the formulation of diagnostic criteria. Initially, Australian (52), American (53), and British (54) researchers separately developed consensus criteria sets for the case definition of the disorder. In 1992 the CDC (55) then proposed the term "chronic fatigue syndrome" to replace numerous previous eponyms, in particular, because this name removed the presumption of a postinfective or any other inflammatory etiology (as was implied in the earlier British nomenclature "myalgic encephalomyelitis"). In 1994, the CDC convened an international study group to formulate the most widely accepted diagnostic criteria (1) (Table 2). These criteria define persons with CFS as presenting with unexplained, persistent, or relapsing fatigue of at least six months' duration, that is not relieved by rest, results in disability, and is accompanied by at least four of eight core symptoms (postexertional fatigue, impaired memory or concentration, unrefreshing sleep, headaches, muscle pain, multijoint pain, sore throat, and tender cervical/axillary lymph nodes). With these criteria an epidemiologic perspective was provided for research into fatigue states. This perspective recognized that although prolonged fatigue syndromes are common and are associated with increased healthcare utilization, such syndromes are likely to be heterogeneous with regard to etiology, and possibly also to pathophysiology (56, 57). These criteria are consistent with symptom constructs described in both population-based samples and clinic studies (56, 58–60). A total of 20 sets of diagnostic criteria for CFS have been published including from the US Institute of Medicine, but it is not clear whether any offer a significant advance over the international consensus criteria, as none has been adequately tested to determine how well they differentiate patients with CFS from patients with other fatigue-related conditions (3, 61). As the symptom set of CFS is intrinsically subjective, an important milestone in improving the diagnostic criteria was the development of instruments to standardize assessment with validated self-report questionnaires, which was recommended by the reconvened international study group in 2003 (62). In addition, a semistructured clinical interview has recently been developed and validated with good sensitivity and specificity in assessing fatigue-related conditions (CFS, acute infection, post-cancer fatigue, major depression) and healthy subjects (63). Interestingly, in this study the symptom domains of fatigue and neurocognitive difficulties were shared across medical and psychiatric boundaries, whereas the key symptoms of major depression, such as anhedonia, were distinguishing.

TABLE 2 Diagnostic criteria for chronic fatigue syndrome (1)

A. Clinically evaluated, unexplained, persistent, or relapsing fatigue that is of new or definite onset; is not the result of ongoing exertion; is not substantially alleviated by rest; and results in substantial reduction in previous levels of occupational, educational, social, or personal activities;

and

B. *Four or more of the following concurrent and persistent symptoms*:

- Impaired short-term memory or concentration
- Sore throat
- Tender cervical or axillary lymph nodes
- Muscle pain
- Multijoint pain without arthritis
- Headaches of a new type, pattern, or severity
- Unrefreshing sleep
- Postexertional malaise lasting more than 24 hours

PROPOSED ETIOLOGIES

The leading hypotheses for the pathophysiological basis of CFS include a unique pattern of infection with a recognized or novel pathogen; an abnormal immune response to a recognized pathogen; a psychologically determined response to infection or other stimulus occurring in "vulnerable" individuals; a metabolic or neuroendocrine disturbance initiated by an unknown trigger; and a neuro-immune disturbance triggered by acute infection (reviewed in 14, 15, 64). Interactions and overlaps between these alternatives are also possible. In addition, a myriad less-extensively evaluated hypotheses exist.

Infection

The potential role of infectious agents in producing chronic fatigue is the proposal that has been most closely examined. This notion arose naturally from the historical observations linking specific infections, such as brucellosis, to a subsequent fatigue state. Further support for this possibility comes from the anecdotal histories that patients with CFS give. These recollections typically describe a "flu-like" illness demarcating the patient's prior good health from the subsequent chronic fatigue state. Unfortunately, these associations are frequently retrospective attributions with uncertain validity, as the expected incidence of symptomatic viral infections in the general population is approximately four annually (65), making chance associations likely. In addition, many reported case series are confounded by the selection bias of referral to specialty clinics *because* of the history of an infective illness (66). Nevertheless, the prospective studies following patients from the onset of acute EBV and other infections provide clear support for the hypothesis that a discrete postinfective fatigue syndrome can be precipitated by specific pathogens (7, 10, 27, 28). Several other viral, and some nonviral, agents have been implicated as apparent triggers for a postinfective fatigue syndrome (Table 1). A noteworthy inclusion in this list is the so-called "post-Lyme borreliosis syndrome" or "chronic Lyme disease," which features fatigue, musculoskeletal pain, and neurocognitive difficulties lasting months or years after primary infection (67), and is unresponsive to prolonged antibiotic therapy (68–70).

Hypothetically, abnormal persistence of the triggering pathogen may underpin postinfective fatigue syndromes. Several early studies examined this possibility in patients with CFS without consideration of the (likely) heterogeneous nature of the triggering agents in such patient groups. Although initial reports suggested "elevated" titers of antibodies directed against organisms like EBV and human herpesvirus-6 (HHV-6) (perhaps implying persistent antigenic stimulation), these findings were refuted in suitably designed case-control studies and discordant twin studies (reviewed in 14, 15).

However, the notion of persistence of the microorganism as a key component of the pathogenesis of postinfective fatigue has been supported by the report of persistent microbial nucleic acids and antigenic, but nonviable, cell residues in patients with postinfective fatigue following serologically documented and appropriately treated Q fever (71, 72). By contrast, attempted PCR amplification of spirochetal DNA from the blood of 1800 patients with post-Lyme borreliosis syndrome was uniformly negative (73). Although, EBV persists naturally in all subjects following primary infection, by establishment of latency in B lymphocytes, the possibility of alterations in viral titer during or following the acute illness was examined in a case-control series of subjects with infectious mononucleosis who developed a postinfective fatigue syndrome in comparison to those who recovered promptly—no difference in cell-associated viral load in the peripheral blood was detected at any time point (74). Thus, there is a growing body of evidence against the role of ongoing, active infection with the triggering pathogen in postinfective fatigue syndrome.

In relation to CFS, the possibility of reactivation of latent HHV-6 infection as a driver for an ongoing immune response, and hence ongoing symptoms, has been examined in several studies, with inconclusive results (75–78). The initial positive report based on viral cultures, augmented by immunofluorescence and PCR detection, demonstrated the presence of HHV-6 in peripheral blood mononuclear cells in a substantially greater proportion of patients than control subjects. However, other case-control studies using both serologic and PCR techniques have yielded conflicting evidence for HHV-6 reactivation (including specific examination of HHV-6A and B) in patients with CFS. Similarly, an association between persistent detection of the enteroviral antigen, VP1, and of enteroviral RNA in muscle biopsies of a subset of patients with CFS (79, 80) was refuted in subsequent reports, including one from the same research group (81–83).

Similarly, searches for a novel pathogen, including retroviruses (84–87) and Borna virus (88, 89), in patients with CFS have been fruitless, with initial positive findings failing to pass the subsequent hurdle of independent confirmation. The most recent example of this novel-pathogen hypothesis was initiated by a report describing the detection of sequences of Xenotropic Murine Leukemia Virus-related Virus (XMRV), a gamma retrovirus, as well as putative infectious virus, in the blood of a majority of patients with CFS (67%), compared to a small proportion (3.7%) of healthy individuals (90). Multiple subsequent studies failed to replicate the finding (91–94), which was ultimately shown to be a chimeric retrovirus generated by passage of human tissue in mice (95).

Given the diversity of specific infections now clearly linked temporally with the onset of CFS, and the essentially negative findings for occult pathogens to date, it is highly unlikely that any single infectious agent will be identified as the cause of CFS.

Immunologic disturbance

The clinical course of most acute infections ranges from asymptomatic, to severe, incapacitating illness. This spectrum of disease is at least partially due to the magnitude of the immune response induced to control the invading organism, rather than to a direct effect of microbial replication. Cytokines, such as the interleukins (IL)-1, IL-6, tumor necrosis factor (TNF)-alpha, and the interferons, are released in a cascade of cellular activation induced by microbial antigens (see Chapter 14). In the context of acute infection, the classical sign of this host response, that is fever, has been shown to be induced by the action of pro-inflammatory cytokines on receptors within the brain (8). Similarly, the severity of the neurobehavioral manifestations of the acute sickness response, including fatigue, musculoskeletal pain, anorexia, somnolence, and mood disturbance, was shown to correlate with production of pro-inflammatory cytokines (96).

Accordingly, the immunologic hypothesis for postinfective fatigue syndrome has proposed that an aberrant and persistent cellular immune response to precipitating infectious agents results in chronic cytokine production, which may directly mediate protracted symptoms (97). Studies of cellular immunity in patients with CFS have produced widely varied results (reviewed in 98). The most consistent findings are of alterations in T-cell responses and reduced natural-killer-cell activity, but these changes have rarely correlated with the clinical condition. Given the diversity of infective and other triggers for the onset and persistence of CFS, these inconclusive findings may reflect underlying heterogeneity in the condition (56, 57). A comprehensive examination of antigen-specific immune responses against the pathogen documented to trigger the onset of the

postinfective fatigue syndrome was undertaken in a longitudinal case-control series of subjects followed from acute EBV infection either into a postinfective fatigue syndrome or to prompt resolution of clinical illness. Although minor differences in the kinetics of both humoral and T-cell responses against latent EBV antigens were documented, these changes did not correlate with the course of illness (74).

Numerous studies of cytokine production have been conducted in patients with CFS, with no consistent alteration detected in serum or cerebrospinal fluid cytokine levels (99–104, reviewed in 105). The possibility of aberrant, exercise-induced cytokine production has also been evaluated with negative findings (105–107). The opportunity to examine cytokine production in response to antigens, derived from the microbe believed to have precipitated the postinfective fatigue syndrome, has been provided by the recognition of a post-Q fever fatigue syndrome. The initial report suggested that increased production of IL-6 was evident in samples from subjects with post-Q fever fatigue when compared to samples from subjects who recovered uneventfully from acute Q fever (108). However, more comprehensive examinations of serum levels and *ex vivo* production of cytokines, in patients with postinfective fatigue syndrome and matched control subjects who recovered promptly after EBV, Ross River virus, or Q fever, did not reveal any significant alterations in cytokine production in those with postinfective fatigue (109, 110).

In summary, although there is evidence of minor immunologic alterations in patients with CFS, the changes are generally inconsistent, do not correlate with disease severity or course, and are not associated with clinically significant consequences, such as infection or malignancy. Cytokine levels do correlate with the acute-phase illness manifestations, including fatigue, but there is no evidence for ongoing cytokine production in the circulation in patients with postinfective fatigue. The possibility of an immunologic disturbance restricted to the central nervous system triggered by the insult of acute infection has not been formally examined.

Given these findings, there is no current role for immunologic testing as a diagnostic tool in the assessment of patients with CFS (1), other than for the detection of alternative medical diagnoses (Fig. 2 and Table 3).

Psychological Disturbance

Approximately 30 to 70% of patients with CFS also meet criteria for the diagnosis of major depression (111–113 and reviewed in 14, 15). This high degree of comorbidity with depression is, in part, an artifact of overlapping symptoms (both disorders list fatigue, sleep disturbance, psychomotor change, cognitive impairment, and mood change as characteristic features). Furthermore, major depression is a common accompaniment of most chronic, disabling medical disorders. Some characteristic clinical features of major depression do help differentiate from CFS (if present), including weight loss, feelings of guilt and suicidal ideation, and observable psychomotor slowing. The mood disturbance described by patients with CFS tends to be of irritability and transient depression rather than the profound loss of interest in, and pleasure from, daily activities (i.e., persistent anhedonia), which is the hallmark of a primary major depression (63).

The presence of concurrent mood disturbance does not demonstrate that depression is the cause of CFS. Studies have therefore sought to determine the rate of premorbid psychiatric disorder and also compared patients with CFS with subjects suffering from other relevant psychiatric, neurologic, and chronic medical illnesses (114). Patients with CFS appear to occupy an intermediate status, having more premorbid and current psychopathology than patients with medical illnesses, but less than patients with overt psychiatric disorders.

The most contentious area of psychiatric comorbidity concerns "somatoform disorders" (i.e., psychological disturbances presenting with physical complaints). Patients with typical somatization disorder complain of a wide range of nonspecific medical symptoms, demonstrate a lifelong pattern of excessive medical treatment, and reject psychological interpretations of their illness. It is clear that analyses of the symptoms presented by patients with CFS in tertiary-referral and primary care settings suggest that a significant minority (up to 32%) of patients have a form of somatization (56, 57, 115, 116).

In relation to postinfective fatigue syndrome, the prospective cohort studies have not found a premorbid (i.e., preinfection) history of depression to be a risk factor for a subsequent fatigue syndrome (10, 11). In addition, formal psychiatric assessment of patients with postinfective fatigue at six months after onset of infection revealed comorbid depression to be rare (11). In combination with the studies of CFS, these data suggest that there may be more than one pathway into the CFS—one via infection and another potentially via mood disorder. Whether the biological processes underlying these proposed pathways are shared or divergent is unknown.

Metabolic or Neuroendocrine Disturbances

Hypotheses of several other pathophysiological disturbances have been explored in CFS, including impaired hypothalamic-pituitary-adrenal (HPA) axis activation (117–121), primary sleep disorder (122–125), and autonomic dysfunction (126–129). In the most exhaustive neuroendocrine study (118), lower mean urinary-free cortisol and reduced-basal evening glucocorticoid levels were found in patients with CFS in comparison to healthy control subjects. In addition, the patients were shown to have an increased adrenocortical sensitivity to administered ACTH, but a blunted response to CRH, consistent with a mild hypothalamic defect in the regulation of adrenal function. Other studies have shown generally concordant findings (reviewed in 130). The alterations in HPA axis performance were shown to differ from those in patients with major depression (in whom relative hypercortisolism and resistance to dexamethasone suppression are evident) (117). The available evidence suggests that these changes in patients with CFS are a consequence of the chronic illness, rather than the cause, as they are absent early in the course of illness and improve following unrelated treatment interventions, such as cognitive-behavioral therapy (see below) (130).

The complaint of unrefreshing sleep is a universal one in patients with CFS, often associated with either hypersomnia or insomnia. Accordingly, exclusion of the prevalent primary sleep pathologies, such as obstructive sleep apnea, is clearly important before applying the label of CFS (122, 131, 132). In the absence of such diagnoses, disturbances of sleep maintenance (i.e., frequent awakenings) are prevalent, and circadian rhythm may also be disturbed (133). The early report of a characteristic sleep abnormality, with the intrusion of high-amplitude alpha waves into the delta pattern of stage III–IV non-REM sleep, has not been reliably demonstrated, nor is it specific for CFS (134, 135). It remains plausible that alterations in sleep-stage transitions and

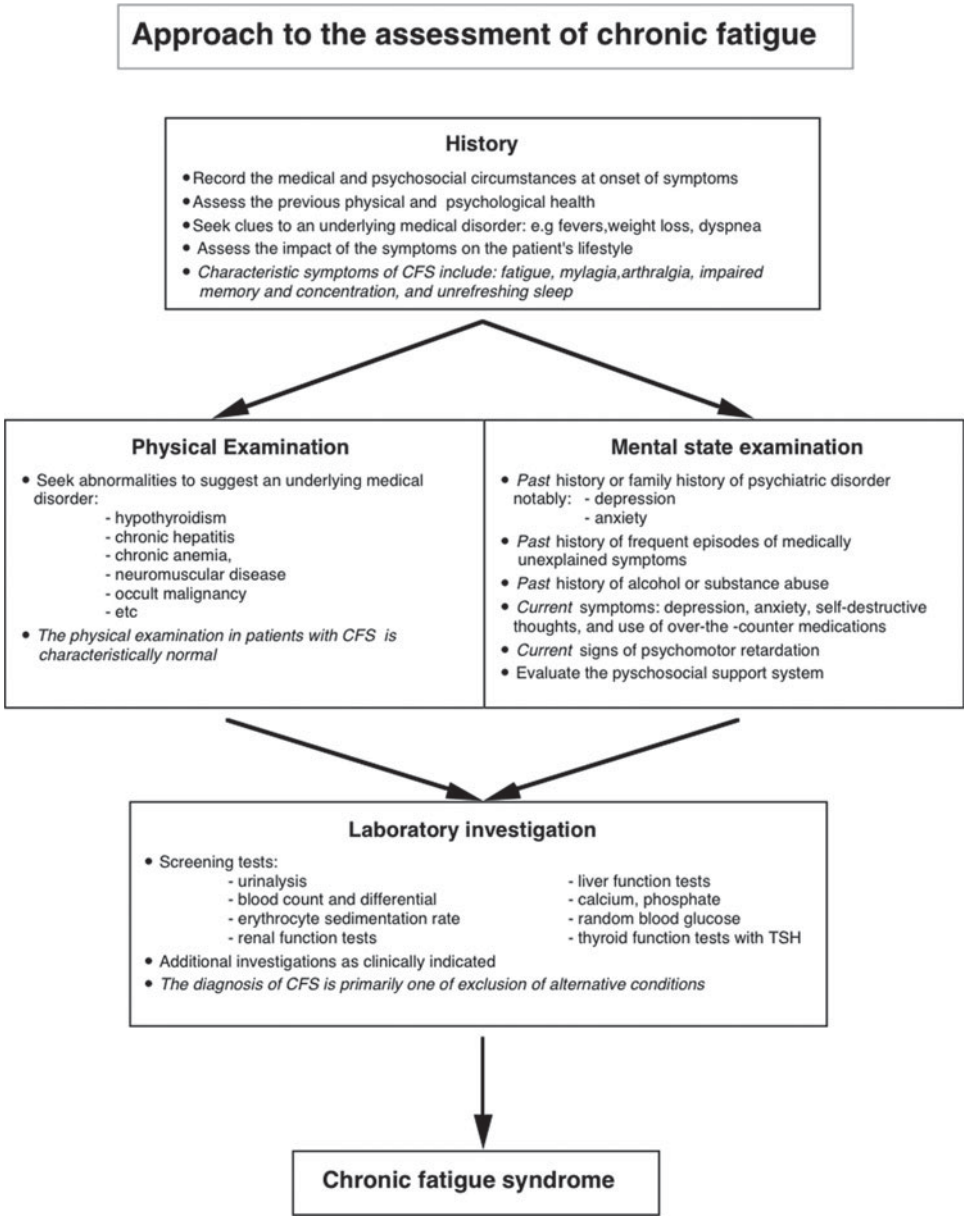

FIGURE 2 Approach to the clinical and laboratory assessment of patients with chronic fatigue. Adapted from reference (157)

associated physiological mechanisms, such as heart rate variability, may be relevant to pathogenesis (136–138 and reviewed in 139, 140, 141).

The potential role of altered autonomic function as a correlate or causative factor in CFS has been explored in several studies. The initial focus was on clinically apparent, neurally mediated hypotension and/or postural tachycardia (126–129). In the initial report, patients were evaluated using a three-stage tilt-table test with the administration of isoproterenol. An abnormal response was documented in 22 of 23 patients (96%) in comparison to 4 of 14 healthy control subjects (29%). Subsequent studies have generally confirmed that a subset of patients with CFS also display features of autonomic instability (142, 143). However, it is unlikely that this phenomenon is causative in CFS, as studies in identical twin pairs, discordant for the illness, have revealed comparable evidence for altered regulation of vasomotor control in the unaffected siblings (126). In addition, appropriate treatment for this condition, in the form of mineralocorticoid supplementation with fludrocortisone to promote salt and water retention, is ineffective in resolving the major symptoms of the disorder (see below) (144). Nevertheless, several case-control studies have revealed reduced heart rate variability in patients with CFS, reflecting reduced parasympathetic (vagal) activity (136, 137, 141, 145, 146, and reviewed in 147). These observations have led to the hypothesis that sensitization within the central nervous system results in abnormally heightened perception of physiological signals from the body in conjunction with autonomic hyperreactivity (reviewed in 148, 149) underpins CFS.

Genetic Predisposition

Family studies of patients with CFS have demonstrated a higher prevalence in relatives, indicating a possible genetic

TABLE 3 Causes of chronic fatigue

Physiologic	***Endocrine disorders***
Increased physical exertion	Hypothyroidism
Inadequate rest	Hyperthyroidism
Sedentary lifestyle	Hyperparathyroidism
Environmental stress (e.g., heat)	Adrenal insufficiency
Drugs	Cushing's syndrome
Medications (e.g., β-blockers)	Hypopituitarism
Alcohol dependence	Diabetes mellitus
Other substance abuse	***Metabolic disorders***
Drug withdrawal	Hypocalcemia
Psychosocial	Hyponatremia
Depression	Chronic renal failure
Anxiety disorder	Chronic liver disease
Somatization disorder	***Infectious diseases***
Dysthymia and grief	Infectious mononucleosis
Pregnancy	HIV/AIDS
Autoimmune disorders	Chronic hepatitis B or C
Systemic lupus erythematosis	Lyme disease
Multiple sclerosis	Syphilis
Rheumatoid arthritis	Tuberculosis
Vasculitides (e.g., Wegener's granulomatosis)	Subacute bacterial endocarditis
	Chronic parasitic infection
Sarcoidosis	Invasive fungal disease
Cardiorespiratory disorders	Obstructive sleep apnea
Chronic airflow limitation (CAL)	Narcolepsy
Cardiac failure	***Neuromuscular disorders***
Silent myocardial infarction	Myopathic diseases
Cardiac arrhythmias	Muscular dystrophies
Mitral valve disease	Myasthenia gravis
Hematologic disorders	Guillain-Barré syndrome
Anemia	***Syndromes of uncertain etiology***
Myeloproliferative disorders	Chronic fatigue syndrome
Occult malignancy	Fibromyalgia (fibrositis)
Sleep disorders	

(or shared environmental) component to the disorder (150). Twin studies also indicate a likely heritable component (151, 152). A genealogical resource from Utah, linking family data and medical diagnoses, revealed a higher relative risk of development of CFS in those with a family history, which was evident between both first-degree (relative risk 2.7) and second-degree relationships (relative risk 2.34) (153).

Genetic polymorphisms confer variations in responsiveness of immunological and neurobehavioral responses (154, 155). During acute infective illnesses, the severity of the key symptoms, including fatigue, has been correlated with the level of production of pro-inflammatory cytokines, including interleukin (IL)-1β and IL-6 (153). Additionally, the severity of mood disturbance in this context was found to be more strongly associated with IL-6 production, whereas fatigue and pain were more closely related to the production of IL-1β (153). These associations suggested that individual cytokines could influence the various aspects of the symptom complex in the acute sickness response to infection. In the postinfective fatigue syndrome, both the severity of the acute illness and the duration of postinfective symptoms (i.e., beyond the initial febrile period) have been associated with functional polymorphisms in the pro-inflammatory cytokine, interferon (IFN)-γ and the anti-inflammatory, IL-10, genes (154). In particular, the high-producing allele of the IFN-γ gene and the low-producing allele of the IL-10 gene were associated with more severe and more prolonged illness. A subsequent study, in which the symptom complex of the acute infective illness and the subsequent postinfective illness was divided into coherent symptom domains, or "endophenotypes," was undertaken to investigate the possibility of individual genetic associations of each symptom domain (155). In this analysis the high-producing allele of the IFN-γ gene was specifically associated with the fatigue domain, whereas the low-producing allele of the IL-10 gene was associated with increased mood disturbance and neurocognitive difficulties. In addition, carriers of the high-producing allele of IL-6 also had increased mood disturbance (155). These findings await confirmation but suggest the genetic predisposition to patterns of pro- and anti-inflammatory cytokine production in the acute illness may partially underpin the susceptibility to a subsequent postinfective fatigue illness and its phenotypic characteristics.

There have been multiple candidate gene-association studies in case-control series of patients with CFS, including investigation of the human leukocyte antigen (HLA) complex, cytokine genes, as well as neurotransmitter pathways, but all of these studies have been underpowered, and none have passed the critical initial benchmark of independent replication (reviewed in 156). Large-scale genetic-association studies of carefully phenotyped patients with CFS may be warranted.

CLINICAL AND LABORATORY ASSESSMENTS

Until a reliable biological marker for the syndrome is available, the key to evaluation of patients presenting with chronic fatigue will continue to include a thorough medical history, physical examination, and detailed assessment of psychological factors (Fig. 2) (157, 158). The medical interview should specifically determine i) whether the fatigue is of recent and discrete onset; ii) the medical and psychosocial circumstances at onset; and iii) the presence of symptoms suggestive of an underlying medical condition to explain the fatigue and related symptoms (e.g., documented fever suggesting infection, weight loss suggesting malignancy, or objective arthritis suggesting autoimmune disease) (1). It is also important to distinguish the characteristics of the phenomenon of fatigue to verify that the primary complaint is not muscle weakness, somnolence, or motivational loss. Patients with CFS characteristically describe profound fatigue, which is precipitated by physical tasks previously achieved with ease and is associated with a protracted recovery period extending over hours or even days.

The physical examination should similarly be directed at detection of signs of unrecognized medical disorders (e.g., goiter, stigmata of chronic liver disease, and neurologic signs of myopathy or multiple sclerosis). The physical examination in patients with CFS should be normal. The psychological evaluation should directly assess current mood, cognitive function, and illness attitudes. Particular attention should be directed towards the identification of serious and treatable anxiety or depressive disorders. Other important historical features include a past or family history of psychiatric disorder, previous episodes of medically unexplained symptoms and excessive healthcare utilization (suggesting a diagnosis of somatization disorder), and excessive use of alcohol or other substances of abuse.

Medical causes of chronic fatigue that should be ruled out include hypothyroidism, chronic hepatitis, anemia, sleep apnea, and side effects of prescribed medications, although many other causes are possible (Table 3). Patients diagnosed with "fibromyalgia" have essentially a synonymous disorder to CFS, differing principally in the prevalence and severity of musculoskeletal pain (159). Psychiatric disorders that commonly present with chronic fatigue include major depression, somatoform disorders, panic and other anxiety disorders, alcohol and substance abuse, and eating disorders.

Despite the wide range of hematologic, immunologic, virologic, psychometric, and neuroimaging investigations that have been conducted, no specific diagnostic test for CFS has emerged. In fact, the clinical heterogeneity of patients diagnosed with CFS makes it highly unlikely that any specific test could emerge (56, 57). Examples of specific tests that do not confirm or exclude the diagnosis of CFS include serological tests for Epstein-Barr virus, human herpesvirus 6, enteroviruses, and *Candida albicans;* detection of nucleic acids of *Mycoplasma sp.* or other microorganisms by PCR; tests of immunity, including T-cell or natural-killer-cell phenotype and functional assays; and neuro-imaging studies, including cerebral magnetic resonance imaging scans or radionuclide studies.

The limited number of screening laboratory tests, which are recommended, are intended for the detection of alternative medical conditions (Table 3) (1). These screening tests should be uniformly negative or normal. If alternative diagnoses are suggested by the clinical history, examination, or screening investigations (e.g., anemia, sleep apnea, or multiple sclerosis), then additional directed investigations (e.g., iron studies, overnight sleep study, or magnetic resonance imaging, respectively) may be warranted. Similarly, if the mental status examination raises the issue of psychiatric disorder, then referral for specialist psychiatric opinion should be sought.

TREATMENT

A wide range of antimicrobial, immunoregulatory, neuroendocrine, metabolic, and antidepressant therapies have been evaluated in randomized, placebo-controlled trials (reviewed in 14, 15, 144, 157, 160–181). While some positive outcomes have been reported, to date, no pharmacologic agent has consistently demonstrated efficacy in repeated, well-designed studies. Several trials have compared an immunologic therapy to placebo and have failed to show reproducible evidence of benefit, including with intravenous immunoglobulin and transfer factor (163, 164, 166, 182), with the possible exception of the Toll-like-receptor 3 agonist, rintatolimod [Poly I: C(12)U], for which limited benefit was evident only in those with severe disease (167, 183). Antiviral drug trials, including with aciclovir and valganciclovir, have failed to show benefit above placebo, although subjects with postinfective fatigue, which had been triggered by well-documented EBV or other herpesvirus infection, were not specifically included (160, 184). Antimicrobial therapy for post-Lyme borreliosis also did not show benefit (68–70). Preliminary findings of benefit in an uncontrolled pilot study of monocycline treatment for chronic fatigue after Q-fever infection (185), await replication in an ongoing randomized controlled trial (186). Similarly, the positive effects of the B-lymphocyte depleting agent, rituximab, await independent confirmation (187, 188).

Four placebo-controlled trials have evaluated a pharmacologic therapy directed at the HPA-axis disturbance by administration of corticosteroids—fludrocortisone and hydrocortisone (172, 189–191). The two trials using low-dose glucocorticoids suggested that this therapy may improve subjective fatigue or the sense of wellness, but at the risk of potentially harmful suppression of adrenal function and risk of other long-term adverse effects (172, 190). The two trials of mineralocorticoids, including one specifically limited to patients with CFS, which was associated with documented neurally mediated hypotension (191), showed no benefit in improving symptoms or functional outcomes (189).

Although antidepressant therapies are commonly suggested for the treatment of CFS, the empirical evidence for their utility is very limited. None of five placebo-controlled trials of antidepressant therapy in patients with CFS have demonstrated a substantive or sustained benefit from the agents studied, which included the monoamine oxidase inhibitors, phenelzine, selegiline, and moclobemide, as well as the serotonin reuptake inhibitor, fluoxetine (168–170, 175, 178).

Studies in patients with the overlapping clinical syndrome of fibromyalgia have demonstrated the benefit of a combination of a low-dose tricyclic antidepressant and a nonsteroidal anti-inflammatory agent, where effects on muscle pain and sleep disturbance are notable (192), arguing for a similar therapeutic trial in patients with CFS in whom pain and sleep disturbance are prominent. Reviews of the evidence-base for management of fibromyalgia endorse the use of pharmacologic agents for pain (antidepressants and centrally active agents, such as gabapentin) and nonpharmacologic interventions (graded exercise, heated pool), consistent with the principles of management of CFS (193–195).

Patient cohorts in such drug treatment trials are likely to be heterogeneous, as a simple consequence of the subjective, prevalent, and nonspecific symptom criteria used to make the diagnosis of CFS. Consequently, any treatment that claims to cure the majority of patients with CFS is likely to be acting via a nonspecific mechanism. As is true in other chronic medical conditions, at least 30% of patients with CFS generally demonstrate improvement in the nonspecific treatment arm of controlled trials (196).

By contrast, numerous randomized controlled trials have established the effectiveness of cognitive-behavioral (197–208) and graded-physical exercise (203, 206, 207, 209–212) in patients with CFS (reviewed in 180, 208, 213, 214). These management approaches link the principles of good clinical care with varying degrees of psychological intervention and functional rehabilitation via graded physical and cognitive activity. The outcomes of such multidisciplinary interventions are best described as improvement in both symptom severity and functional capacity but not cure (215).

Given the likelihood of spontaneous improvement in patients with chronic fatigue, controlled-treatment trials are essential for all proposed therapeutic modalities. Prevention of secondary medical and psychological morbidity due to prolonged rest and social isolation should be emphasized. Nonspecific aspects of good clinical management are effective and include careful medical and psychological evaluation, judicious use of investigations and specialist referral, consistent and empathic interactions with the patient over the course of the illness, and the encouragement of a rehabilitation approach. Irrespective of attitudes to etiology, the patient should be encouraged to incorporate the widest possible view of the role of medical, psychosocial, and rehabilitative strategies to promote recovery.

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Section II

The Agents

Poxviruses

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Smallpox has long been known as a severe human disease (1, 2) and was already endemic in India 2,000 years ago before spreading to China and Japan in the east and Europe and North Africa in the West by about 700 A.D. It was introduced to the Caribbean with the African slave trade in 1518 and thence to Mexico in 1520, taking a terrible toll on the totally nonimmune Amerindians. Repeated introductions from Europe and, to a lesser extent, from Africa, into North America occurred from 1617 onward. With the discovery of vaccination by Jenner in the latter part of the 18th century (3), the disease was brought under control first by local initiatives, which became national, and finally global. Following the last identified case in 1977, the world was certified free of smallpox by the World Health Assembly in May 1980 (2). The subsequent drawdown of vaccine stockpiles and cessation of childhood vaccination programs have increased the vulnerability of the human population to a devastating smallpox epidemic and increased the threat of variola virus (VARV) as a bioweapon. This unintended consequence of the most successful vaccination program in history was exploited by the former Soviet Union, which weaponized VARV in contradiction to the 1972 Biological and Toxin Weapons Convention (4, 5). This occurrence raised concerns that other rogue nations or terrorist groups could also develop VARV or monkeypox virus (MPXV) as a bioweapon.

Monkeypox is a disease that is clinically almost identical to smallpox. The monkeypox virus (MPXV) was discovered as a disease-causing agent of laboratory primates in Copenhagen, Denmark, in 1958 (6), and it caused several other outbreaks in captive primates before it was recognized as the cause of a smallpox-like zoonotic disease in West and central Africa in 1970. The incidence of monkeypox is on the rise in central Africa (7) and caused an outbreak in the United States. It is important to note that the names of many of the orthopoxviruses were based on the species where disease was initially seen and thus are not a species-specific disease. For example, monkeypox virus and cowpox virus obtained their names because the pox infection was first observed in monkeys and cows, respectively.

VIROLOGY

Classification

The poxviruses (family *Poxviridae*) are one of the largest and most complex of all viruses (8, 9). The subfamily *Chordopoxvirinae* (poxviruses of vertebrates) contains eleven genera (10). Human infections have been caused by 13 poxvirus species belonging to four genera, the majority of which cause zoonotic infections (Table 1). The orthopoxvirus pathogens are the best studied, and variola virus (VARV) is the most important species, as it caused a severe human disease known as smallpox. While variola major caused severe disease and death, a second type, called variola minor, caused much lower mortality. Comparisons between the variola major and variola minor genomes show that these viruses have >95% identity of most of the genes (one-third of the genome is 100% identical) (11). It is speculated that the difference in virulence is due to nucleotide additions and deletions resulting in alterations of gene expression and protein truncations.

Vaccinia virus (VACV) and cowpox virus (CPXV) are related but distinct orthopoxviruses that cause human infections. CPXV is the agent that Jenner used to prove the efficacy of vaccination against smallpox; however, VACV was subsequently used for at least the last 100 years to vaccinate against smallpox. Human CPXV infections are rare and are usually acquired from the domestic cat. A VACV ancestor may originally have been maintained in nature in horses, as a comparative genome-sequencing study placed a poxvirus, isolated in 1976 from a diseased Mongolian horse, as a member of the VACV clade of orthopoxviruses (12) with recombination events leading to the virus we call VACV (13). In addition to vaccination and its complications, human infections with VACV result from contact with vaccinees or domestic animals persistently infected with VACV as a result of the smallpox eradication programs (cantagalo and buffalopox viruses) (14–17). VACV and two members of the genus *Avipoxvirus* (fowlpox virus and canarypox virus) have been used as vectors for the production of novel vaccines and as oncolytics for cancer therapy (18–20). Recently, a newly discovered orthopoxvirus (GCP2013) was identified in the country of Georgia in a cow herder who developed chronic painful skin lesions (21).

The remaining human poxvirus pathogens belong to three genera: five in *Parapoxvirus*, two in *Yatapoxvirus*, and one in *Molluscipoxvirus* (Table 1). Orf virus (ORFV) causes orf (synonyms: contagious pustular dermatitis, contagious ecthyma, and scabby mouth), a disease of sheep. Pseudocowpox (PCPV; synonyms: milker's nodule and paravaccinia) and bovine pustular stomatitis (BPSV) viruses

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TABLE 1 Poxviruses that can cause disease in humans

Genus	Species	Distribution	Reservoir	Disease in humans
Orthopoxvirus	Variola virus	Was worldwide	Humans (before 1977)	Smallpox, specifically human, generalized disease
	Monkeypox virus	Central and West Africa	Squirrels, monkeys	Smallpox-like disease; a zoonosis
	Vaccinia virus	Worldwide	Laboratory virus	Used for smallpox vaccination, localized lesions
	Buffalopox virus, variant of vaccinia virus	India (Egypt, Indonesia)	Buffaloes, rodents	Localized pustular skin lesions
	Cantagalo virus, variant of vaccinia virus	Brazil	Cattle	Localized pustular skin lesions
	Cowpox virus	Europe, western Asia	Wild rodents	Localized pustular skin lesions; a rare zoonosis
	Novel orthopoxvirus (Georgia)	Country of Georgia	Not yet defined	Localized pustular skin lesions; a very rare zoonosis
Parapoxvirus	Orf virus	Worldwide	Sheep	Localized nodular skin lesions; a rare zoonosis
	Bovine papular stomatitis virus	Worldwide	Cattle	Localized nodular skin lesions; a rare zoonosis
	Pseudocowpox virus	Worldwide	Cows	Localized nodular skin lesions; a rare zoonosis
	Sealpox virus	Worldwide	Seals	Localized nodular skin lesions; a rare zoonosis
	Novel parapoxvirus (United States)	Tennessee and Tanzania (?)	Unknown	Localized nodular skin lesions; presumed zoonosis
Yatapoxvirus	Tanapoxvirus	Tropical Africa	Unknown	Localized nodular skin lesions; a rare zoonosis
	Yaba monkey tumor virus	West Africa	Unknown, possibly monkeys	Localized nodular skin lesions; rare accidental infections
Molluscipoxvirus	Molluscum contagiosum virus	Worldwide	Humans	Few or many nodular lesions; worse in immunodeficiency

cause diseases of cattle (22). Very rarely, humans may be infected with seal parapoxvirus (23). Recently, a newly discovered parapox-like virus (2013_13 and 2013_37) was identified as causing an infection in an immunocompromised patient who lived in Tennessee and an immunocompetent patient who had traveled to Tanzania (24). While both had contact with animals, the origin of the virus is unknown but is presumed to be a zoonosis. Tanapox virus (TANV) and Yaba monkey tumor poxvirus (YMTV) are rare causes of zoonotic diseases mainly in Africa. Molluscum contagiosum virus (MCV) causes a common cosmopolitan human disease and is the only poxvirus currently maintained in the human population without a zoonotic host (25).

Structure of Virus

Poxvirus virions appear to be oval or brick-shaped structures of about 200 to 400 nm in length with axial ratios of 1.2 to 1.7. The structure of VACV (Fig. 1A and D) is characteristic of that of all the poxviruses that infect humans, except those belonging to the genus *Parapoxvirus* (Fig. 1B and E). The outer membrane of orthopoxviruses consists of tubular lipoprotein subunits arranged rather irregularly, whereas parapoxviruses have a regular spiral structure. The membrane encloses a dumbbell-shaped core and two "lateral bodies." The core contains the viral DNA and associated proteins.

The double-stranded DNA poxvirus genome is 130 to 375 kbp in length and codes for 150 to 300 proteins, depending on the species (see Table 2 for examples of types of proteins encoded by orthopoxviruses). The genomes of a large number of poxvirus species, including 51 isolates of VARV, have been completely sequenced (http://www.viprbrc.org). The genomic organization of the prototypic orthopoxvirus, VACV, is displayed in Figure 2. Orthopoxvirus virions contain a hemagglutinin (A56R, Table 2), and estimates of virus phylogeny based on hemagglutinin and A-type inclusion body protein gene sequences agree well with those based on restriction endonuclease maps, permitting the use of PCR analyses of these genes as the assay of choice for identifying orthopoxvirus species (28–30). Assignment of a poxvirus to a particular taxonomic group is based on extensive analyses of virus genomic sequences. The DNA sequence of strains of an orthopoxvirus species vary by no more than 2%. The orthopoxvirus species vary among themselves by 2 and 13%. The species of the orthopoxvirus genus differ from species of other genera by between 25 and 54%.

There is broad cross-neutralization and cross-protection between viruses belonging to the same genus but little to none between viruses of different genera; in laboratory experiments genetic recombination occurs readily between viruses of the same genus but rarely between those of different genera (31).

Biology

Replication Strategy

Poxviruses are enveloped viruses and thus require the viral envelope to fuse with host cell membranes to gain entry into

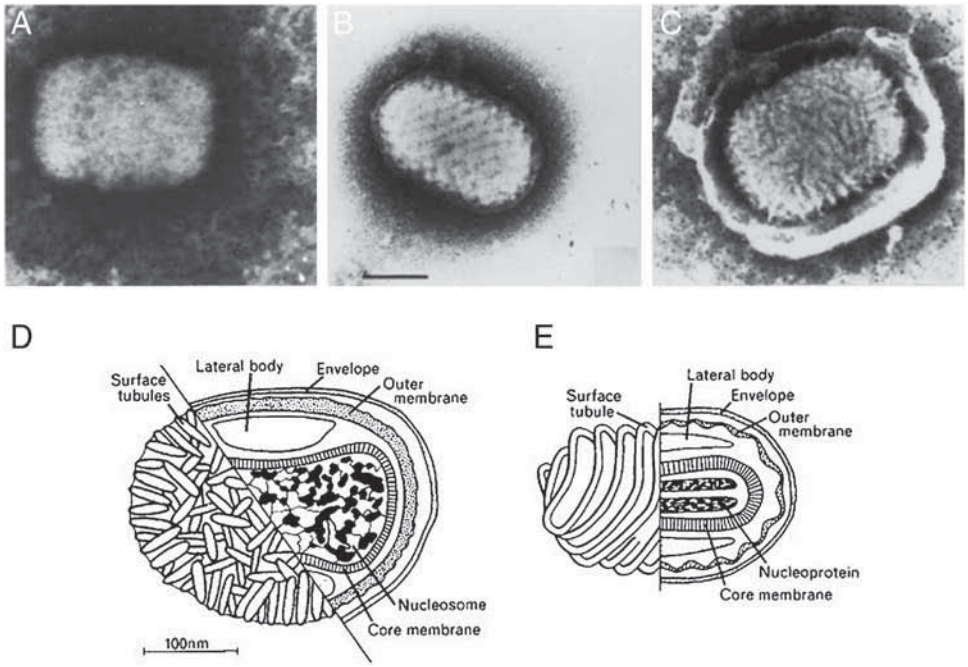

FIGURE 1 Structure of virions of poxviruses that cause human infections. Shown are negatively stained preparations of orthopoxvirus virions (cowpox, monkeypox, vaccinia, and variola viruses) (A), parapoxvirus virions (ORFV, BPSV, PCPV, and sealpox viruses) (B), and yatapoxvirus virions (TANV and YMTV) (C). The virions of orthopoxviruses and yatapoxviruses have similar morphologies, but those of yatapoxviruses always have two membranes. Virions of the parapoxviruses are smaller and have a distinctive regular-surface structure. (D) Diagram of the structure of the virion of VACV. The viral DNA and several proteins within the core are organized as a nucleosome. The core has a 9-nm-thick shell with a regular subunit structure. Within the virion, the core assumes the shape of a dumbbell because of the large lateral bodies, which are, in turn, enclosed within a protein shell about 12 nm thick—the first membrane, the surface of which appears to consist of irregularly arranged surface tubules, which, in turn, consist of small globular subunits. Mature virus released by exocytosis (extracellular virus) is enclosed within a second membrane (envelope) acquired from the Golgi. This membrane contains host-cell lipids and several unique virus-specific polypeptides not found in the intracellular mature virus outer membrane. Most virions remain cell-associated and are released by cellular disruption, without the second membrane. (E) Diagram of the structure of the virion of ORFV. The first membrane consists of a single long tubule that appears to be wound around the particle. In negatively stained preparations (B) both sides are visible, giving a characteristic criss-cross appearance. The second membrane is usually closely applied to the surface of the first membrane. Bar = 100 nm.
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the cell. While a number of cellular attachment factors have been identified, a specific cell receptor(s) that initiates the fusion event has not been identified. Many of the viral proteins involved in attachment and entry are targets of neutralizing antibodies. Viral membrane fusion is dependent on a membrane fusion complex that is made up of 11 to 12 viral proteins (32). Entry can occur at the plasma membrane or within endosomes after micropinocytosis. After entry, the replication of poxviruses occurs in the cytoplasm, and poxviruses encode dozens of enzymes required for transcription and replication of the viral genome, several of which [including DNA-dependent RNA polymerase, poly (A) polymerase, capping enzyme, methylating enzymes, and transcription factor] are carried in the virion itself (Fig. 3) (8). The viral core is released into the cytoplasm after fusion of the virion with the plasma or endosomal membrane. Transcription is initiated by the viral transcriptase, and functional capped and polyadenylated mRNAs are produced minutes after infection. The polypeptides produced by translation of these mRNAs complete the uncoating of the core, and transcription of about 100 early genes, distributed throughout the genome, occurs before viral DNA synthesis begins. Early proteins include DNA polymerase, thymidine kinase, and several other enzymes required for replication of the genome.

With the onset of viral DNA replication there is a dramatic shift in gene expression. Transcription of intermediate and late genes is controlled by binding of specific viral proteins to characteristic promoter sequences. Virion assembly occurs in circumscribed areas of the cytoplasm, where spherical immature particles assembling on cellular membranes can be visualized by electron microscopy. The immature particle incorporates DNA and additional proteins during morphogenesis to the mature virus. Most mature virus remains in the cytoplasm and is released on cell death; however, some moves to the Golgi complex, where it is wrapped in a double membrane, transported to the plasma membrane, and released by exocytosis with the loss of one of the Golgi acquired membranes to produce extracellular virus (that has an additional viral membrane) (Fig. 3). Both mature virus and extracellular virus particles are infectious, but extracellular virus particles appear to be important in virus spread through the body.

Poxviruses encode a large number of proteins that enhance the ability of the virus to replicate efficiently and spread within the animal host (8). A number of these gene products are important for optimal replication in differentiated and nondividing cell types, and some are involved in the blockade of cellular apoptosis pathways (33). Others are immunosubversive proteins that are secreted mimics of host

TABLE 2 Examples of genes/proteins encoded by Orthopoxviruses

Gene name[1] in VACV strain WR	Gene name[2] in VACV strain Copenhagen	Description of encoded protein[3]
Involved in viral DNA replication		
VACWR138	A18R	DNA helicase; effects elongation and termination of postreplicative viral transcription
VACWR141	A20R	DNA polymerase processivity factor that interacts with D4
VACWR142	A22R	a late protein essential for concatemer resolution. An E. coli RuvC-like protein with Holliday junction resolvase activity
VACWR155	A32L	putative ATPase involved in DNA packaging
VACWR174	A48R	thymidylate kinase
VACWR176	A50R	ATP-dependent DNA ligase
VACWR115	D10R	contains mutT-like motif of NTP-phosphohydrolase for DNA repair
VACWR109	D4R	uracil glycosylase that also acts as a DNA polymerase processivity factor that interacts with A20
VACWR110	D5R	DNA-independent NTPase essential for DNA replication
VACWR114	D9R	contains mutT-like motif of NTP-phosphohydrolase for DNA repair
VACWR065	E9L	viral DNA polymerase
VACWR056	F17R	putative DNA-binding phosphoprotein in virus core
VACWR041	F2L	dUTPase, expressed early, nonessential in tissue culture, involved in nucleotide metabolism
VACWR043	F4L	ribonucleotide reductase small subunit
VACWR104	H6R	topoisomerase type IB
VACWR070	I1L	DNA-binding core protein
VACWR072	I3L	ssDNA-binding phosphoprotein
VACWR073	I4L	ribonucleotide reductase large subunit
VACWR094	J2R	thymidine kinase; common site to insert foreign genes by homologous recombination
Involved in viral RNA transcription		
VACWR119	A1L	viral late gene transcription factor (VLTF)-2
VACWR143	A23R	45kDa large subunit of viral intermediate gene transcription factor (VITF)-3
VACWR144	A24R	DNA-dependent RNA polymerase subunit (rpo132)
VACWR152	A29L	DNA-dependent RNA polymerase (rpo35)
VACWR120	A2L	viral late gene transcription factor (VLTF)-3
VACWR124	A5R	DNA-dependent RNA polymerase subunit (rpo19)
VACWR126	A7L	82kDa large subunit of viral early gene transcription factor (VETF)
VACWR127	A8R	32kDa small subunit of viral intermediate gene transcription factor (VITF)-3 used for intermediate gene expression
VACWR116	D11L	ATPase, nucleoside triphosphate phosphohydrolase-I, NPH-I; transcription elongation, termination, release factor
VACWR117	D12L	small subunit of mRNA capping enzyme; viral transcription termination factor (VTF)
VACWR106	D1R	large subunit of mRNA capping enzyme; transcription termination factor (VTF)
VACWR111	D6R	70kDa small subunit of viral early gene transcription factor (VETF)
VACWR112	D7R	DNA-dependent RNA polymerase subunit (rpo18)
VACWR057	E1L	poly-A polymerase catalytic subunit (VP55)
VACWR059	E3L	double-stranded RNA binding protein; inhibits antiviral activities of interferon; host-range determinant; host defense modulator
VACWR060	E4L	DNA-dependent RNA polymerase subunit (rpo30), viral intermediate-gene transcription factor (VITF)-1
VACWR080	G2R	late transcription elongation factor
VACWR083	G5.5R	DNA-dependent RNA polymerase subunit (rpo7)
VACWR086	G8R	viral late gene transcription (VLTF)-1
VACWR099	H1L	tyr/ser protein phosphatase VH1, is essential for viral transcription in vivo and in vitro
VACWR102	H4L	tightly associated with DNA-dependent RNA polymerase, aids early-stage transcription preinitiation and termination (RAP94)

(*Continued*)

TABLE 2 Examples of genes/proteins encoded by Orthopoxviruses (*Continued*)

Gene name[1] in VACV strain WR	Gene name[2] in VACV strain Copenhagen	Description of encoded protein[3]
VACWR103	H5R	viral late gene transcription factor (VLTF)-4; substrate of B1R kinase
VACWR077	I8R	an encapsidated 72kDa protein, with NTPase and RNA helicase activity, essential for early transcription
VACWR095	J3R	multifunctional poly-A polymerase subunit, cap methyltransferase, and transcription elongation factor
VACWR096	J4R	DNA-dependent RNA polymerase subunit (rpo22)
VACWR098	J6R	DNA-dependent RNA polymerase subunit (rpo147)
VACWR091	L4R	ssDNA/ssRNA binding protein involved in early mRNA regulation
Involved in viral cytoplasmic disulfide bond formation		
VACWR121	A2.5L	S-S bond formation pathway; CxxxC links SH-oxidase E10R and thioredoxin G4L
VACWR066	E10R	S-S bond formation pathway; sulfhydryl oxidase, substrates L1R/F9L
VACWR081	G4L	S-S bond formation pathway; thioredoxin-like
Involved in formation and/or part of mature virus (MV)		
VACWR129	A10L	precursor p4a of core protein 4a, complexes with A4L
VACWR131	A12L	core protein
VACWR132	A13L	MV membrane protein; target of neutralizing antibody
VACWR134	A14.5L	nonessential hydrophobic IV and MV membrane protein; deletion attenuates virus virulence
VACWR133	A14L	repression of 90 aa A14 membrane phosphoprotein arrests virion assembly prior to/at crescent formation; interacts with A17; F10 and H1 substrate
VACWR136	A16L	component of the poxvirus multiprotein EFC, soluble myristylprotein
VACWR137	A17L	MV membrane protein required for morphogenesis
VACWR140	A21L	component of the poxvirus multiprotein EFC
VACWR149	A26L	MV protein required for incorporating MV into A-type inclusion body; involved in MV attachment to cell surface
VACWR150	A27L	MV surface protein; roles in MV-cell attachment, fusion, and microtubule transport; target of anti-MV neutralization
VACWR151	A28L	component of the poxvirus multiprotein EFC, target of neutralizing antibody
VACWR153	A30L	vaccinia core protein component of the seven protein complex required for virosomal uptake by viral crescents
VACWR122	A3L	p4b precursor of core protein 4b
VACWR123	A4L	39kDa core protein complexes with core protein p4a/4a
VACWR128	A9L	MV membrane protein required for morphogenesis
VACWR107	D2L	virion core protein
VACWR108	D3R	virion core protein
VACWR113	D8L	MV membrane protein binds cell surface chondroitin; may effect viral entry
VACWR118	D13L	rifampicin target associates with inner surface immature virus membrane
VACWR044	F5L	membrane protein that has a role in plaque morphology in a subset of cell lines
VACWR048	F9L	peripheral partner of EFC, S-S bond formation pathway; thiol substrate
VACWR079	G3L	component of the poxvirus multiprotein EFC
VACWR087	G9R	component of the poxvirus multiprotein EFC, myristylprotein
VACWR100	H2R	component of the poxvirus multiprotein EFC
VACWR101	H3L	MV heparin binding surface protein involved in MV maturation; neutralizing antibody target
VACWR071	I2L	component of the poxvirus multiprotein EFC
VACWR076	I7L	viral core cysteine proteinase
VACWR097	J5L	component of the poxvirus multiprotein EFC
VACWR088	L1R	MV membrane protein target of neutralizing antibody; S-S bond formation pathway thiol substrate; myristylprotein
VACWR092	L5R	component of the poxvirus multiprotein EFC

(*Continued*)

TABLE 2 Examples of genes/proteins encoded by Orthopoxviruses (*Continued*)

Gene name[1] in VACV strain WR	Gene name[2] in VACV strain Copenhagen	Description of encoded protein[3]
Involved in formation and/or part of extracellular virus (EV)		
VACWR156	A33R	EV membrane phosphoglycoprotein associates with A36R; involved in CEV-cell adherence and actin tail formation
VACWR157	A34R	EV glycoprotein involved in CEV cell adherence and actin tail formation
VACWR159	A36R	transmembrane phosphoprotein that is required for EV formation, but not incorporated into EV membrane
VACWR181	A56R	EV type-I membrane glycoprotein; inhibits cell fusion and viral superinfection; also named hemagglutinin
VACWR187	B5R	EV type-I membrane glycoprotein; required for trans-Golgi/endosomal membrane-wrapping of MV
VACWR051	F12L	involved in plaque and EV formation; on IEV, not CEV, IV, or MV
VACWR052	F13L	palmytilated EV membrane protein; phospholipase motif, required for IEV formation; target of the anti-poxvirus drug ST-246
VACWR033	K2L	serine protease inhibitor–like (SPI-3); prevents cell-cell fusion; host range determinant; host defense modulator
Host range genes		
no ortholog	B23R	host range polypeptide
no ortholog	B24R	host range protein
no ortholog	C18L	host range protein
VACWR021	C7L	host range protein for growth in cell culture; possible host defense modulator
VACWR032	K1L	host range gene needed for viral replication in rabbit cells and is capable of complementing for C7L function in human cells
Viral immune evasion or proteins that interact with host proteins		
VACWR158	A35R	inhibits MHC class II antigen presentation
VACWR165	A40R	C-type lectin-like type-II membrane protein; deletion attenuates intradermal lesion in Vac-mouse model; host defense modulator
VACWR166	A41L	secreted glycoprotein; deletion causes more severe lesion and enhanced viral clearance in VAC-mouse skin model; host defense modulator
VACWR170	A44L	hydroxysteroid dehydrogenase; deletion attenuates intradermal lesion in VAC-mouse model; host defense modulator
VACWR172	A46R	Toll/IL1-receptor [TIR]-like; suppresses TIR-dependent signal transduction; host defense modulator
VACWR178	A52R	Toll/IL1-receptor [TIR]-like; suppresses TIR-dependent signal transduction; host defense modulator
VACWR179	A53R	gene fragment, secreted TNF-receptor-like protein; CrmC, intact in some Vac strains; host defense modulator
VACWR195	B13R	serine protease inhibitor–like (SPI-2, CrmA); inhibits Fas-mediated apoptosis, IL-1 convertase, lipoxygenase pathway
VACWR197	B16R	interleukin-1-beta-inhibitor; prevents febrile response in Vac-mouse intranasal model; host defense modulator
VACWR199	B18R	Interferon-alpha/beta-receptor-like secreted glycoprotein; host defense modulator
VACWR183	B1R	B1 is an early ser/thr kinase needed for ongoing viral DNA replication capable of phosphorylating the viral H5 and cellular ribosomal proteins
VACWR218	B29R	chemokine-binding protein; host defense modulator [found in the ITR]
VACWR190	B8R	soluble interferon-gamma receptor–like; host defense modulator
VACWR009	C11R	secreted epidermal growth factor–like; binds EGF receptor [found in the ITR]
VACWR205	C12L	serine protease inhibitor–like SPI-1; host defense modulator
VACWR001	C23L	chemokine-binding protein; host defense modulator [found in the ITR]
VACWR025	C3L	secreted complement binding protein; host defense modulator
VACWR034	K3L	interferon resistance; host defense modulator
VACWR028	N1L	virokine; host defense modulator

(*Continued*)

TABLE 2 Examples of genes/proteins encoded by Orthopoxviruses (*Continued*)

Gene name[1] in VACV strain WR	Gene name[2] in VACV strain Copenhagen	Description of encoded protein[3]
VACWR013	no ortholog	interleukin-18-binding protein; host defense modulator
VACWR210	no ortholog	secreted epidermal growth factor–like; binds EGF receptor [found in the ITR]
Miscellaneous		
VACWR162	A38L	CD47-like putative membrane protein
VACWR167	A42R	profilin-like
VACWR168	A43R	Conserved in all orthopoxviruses, but dispensable type-I membrane glycoprotein and not part of EV formation
VACWR171	A45R	inactive Cu-Zn superoxide dismutase–like in virion
VACWR194	B12R	ser/thr protein kinase–like
VACWR049	F10L	ser/thr kinase that phosphorylates viral proteins

[1]Gene names in VACV strain WR are named sequentially from left side of the genome to the right side (1 to 218).

VACV strain Copenhagen was the first VACV genome completely sequenced (193). The gene names are based on the HindIII restriction that digests the ~200 Kbp genome into 15 fragments. The largest fragment is A, next largest is B, etc. Gene names are then numbered within each HindIII fragment and are denoted with an L or an R if the gene is facing to the left or the right.

[2]Description of protein function adapted from poxvirus.org.

[3]CEV, cell-associated extracellular virus; Crm, cytokine response modifier; EV, extracellular virus; IEV, intracellular extra-enveloped virus); ITR, inverted terminal repeat; IV, immature virus; MV, mature virus.

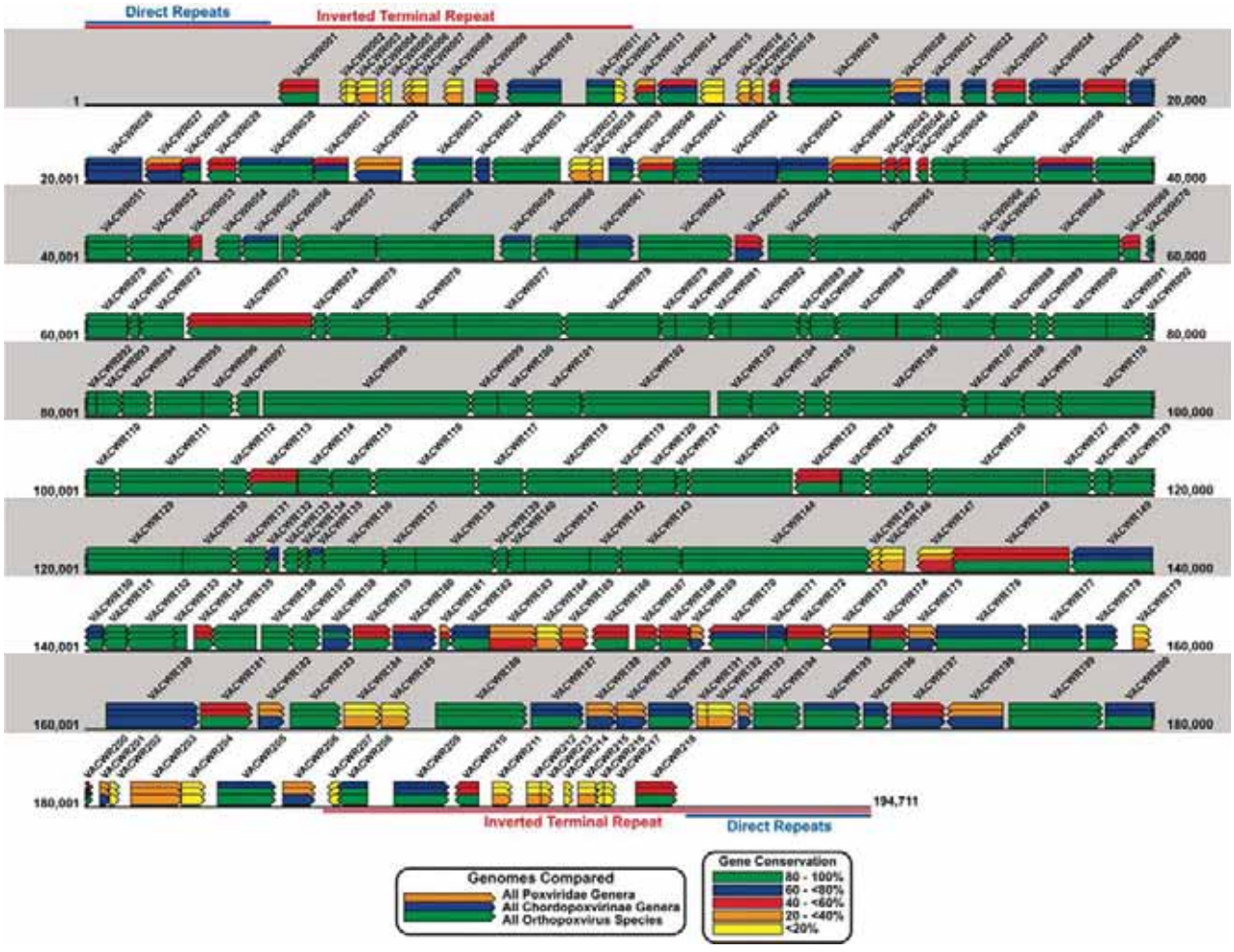

FIGURE 2 Schematic representation of the genome of the WR strain of VACV. The genome is a linear double-stranded molecule with terminal hairpins, inverted terminal repeats, and a series of direct repeats within the inverted repeats. Each overlapping bar indicates gene conservation between the WR strain and all poxviruses, vertebrate poxviruses, and orthopoxviruses. The bars are color-coded according to the percentage of gene conservation across the indicated taxa.
Reprinted from reference (27) with permission.

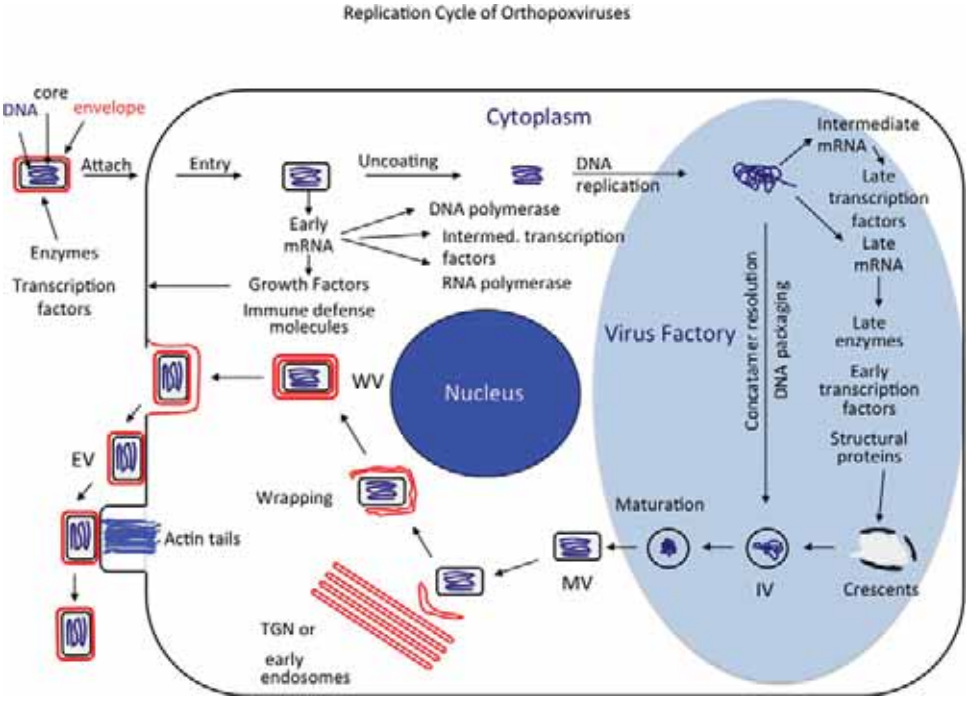

FIGURE 3 Diagram illustrating the replication cycle of vaccinia virus, the prototype orthopoxvirus. IV, immature virus; MV, mature virus; WV, wrapped virus; EV, extracellular virus.
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ligands, regulators, or receptors. These include homologs of epidermal growth factor and binding proteins for complement regulatory proteins C3 and C4, interleukin 1, interleukin 18, tumor necrosis factors alpha and beta, and type I and type II interferons (34–36).

Host Range

The animal host range of poxviruses that infect humans is narrow for VARV and MCV and relatively broad for VACV, CPXV, and MPXV. VARV has not been shown to cause significant disease in immunocompetent animal models except in nonhuman primates, in which lethal infections occur under conditions not characteristic of natural infection (37, 38). Aerosol challenge of cynomolgus macaques with up to $10^{8.5}$ PFU of VARV results in an inapparent infection (37), whereas intravenous challenge with 10^{8} PFU of VARV results in replication and the development of skin lesions, but is usually not lethal. Lethal disease is achieved 3 to 6 days following intravenous administration of 10^{9} PFU. While these models do not recapitulate the natural pathogenesis of human VARV, which is initiated by deposition of a small number of virions on the respiratory mucosa, followed by the appearance of rash (10 to 12 days later), and death (20 to 22 days later) or recovery, they provide ability to test vaccines and therapeutics against variola virus in an animal model (39).

The ecology of MPXV is still not completely understood with respect to reservoir and incidental hosts (40, 41). Seroprevalence studies suggest that there may be several animal species serving as MPXV reservoirs in nature. For example, two genera and six species of squirrels and five genera and nine species of nonhuman primates have MPXV-specific antibodies. Virus has been isolated from an animal in nature only once, and the source was a diseased squirrel of the species *Funisciurus anerythrus* (42). However, cell culture and PCR assays demonstrated MPXV in two *Funisciurus* spp. (rope squirrel), one *Cricetomys* sp. (giant pouched rat), and three *Graphiurus* spp. (African dormouse) from a 2003 shipment of African rodents to the United States (43). These observations suggest the natural ecology of MPXV to be a complex interaction of reservoir hosts and incidental species. This broad host range is a cause for concern, as it may facilitate the adaptation of MPXV to new hosts in new regions. Laboratory studies have demonstrated that cynomolgus macaques (44), prairie dogs (45), and ground squirrels (46) can serve as experimental hosts.

CPXV has a very broad host range in nature. The virus is maintained in wild rodents in western Eurasia, in particular, voles and wood mice. Short-tailed field voles have been proven to be the reservoir host in Great Britain (47). Cows are merely incidental hosts, as are rats, dogs, zoo animals (elephants, lions, cheetahs, pumas, panthers, and jaguars), and domestic cats (48, 49). The domestic cat is the frequent liaison host of human infections (50). In the laboratory, the mouse model is most often used to study CPXV.

VACV infects domesticated livestock species, possibly as a result of the smallpox vaccination campaign in which various animals were purposely used for vaccine production or inadvertently infected by contact with vaccinees (51). In Holland in 1963, 8 out of 36 outbreaks of "cowpox" in milking cows were found to be caused by VACV, and the rest were caused by authentic CPXV (52). In 1985, VACV was isolated from scabs taken from pox lesions on buffaloes in five different districts of Maharashtra State, India (53), and was also observed in Egypt and Indonesia, where water buffaloes (*Bubalus bubalis*) are used as domestic animals (54). Buffalopox outbreaks continued through 1996, with human infections and possible subclinical disease in areas of endemicity (54). There have been ongoing outbreaks of VACV in cattle and dairy workers based in Minas Gerais State, Brazil (55).

Similarly, in 1932, VACV appeared to adapt to a rabbit host, causing a new disease that was highly lethal and transmissible to contact rabbits by airborne infection (56). The isolated agent was named rabbitpox virus, and sequencing of its genome confirmed it as a strain of VACV (57). VACV replicates to various degrees in most tested animal models.

MCV has the narrowest host range of any poxvirus and has similarities in this regard to human papillomavirus (**see Chapter 29**): no experimental animals have been shown to support its growth.

Growth in Cell Culture

Although the host range of human poxvirus pathogens can be quite narrow, all the orthopoxviruses replicate in a broad range of fibroblast or epithelial cell lines, including HeLa, BS-C-1, and Vero cells. Infection causes the cell to round and detach from the substrate, which is the basis for the plaque assay used to measure virus infectivity. Cytoplasmic inclusion bodies can be detected with appropriate histologic stains. MCV appears to replicate only in the human keratinocyte and has yet to be cultivated in cultured cells. MCV from skin lesions has been propagated in human foreskin xenografts (58, 59).

Inactivation by Physical and Chemical Agents

Poxvirus infectivity is relatively stable in the environment compared to other enveloped viruses, and stability is measured in days or weeks, although not years (2). Since all poxvirus species likely share similar sensitivities to physical and chemical agents, data acquired with one poxvirus species can be applied to another. VACV infectiousness is adversely affected by both high temperatures and high humidity. VACV (10^7 pock-forming units on chorioallantoic membrane/ml) sprayed in aerosols at room temperature (21 to 23°C) maintained 46 and 24% of the initial infectivity in low (18 to 19%) and high (82 to 84%) relative humidity, respectively, over a 4-h period (60). Higher temperatures (31.5 to 33.5°C) do not reduce infectivity further at low humidity, whereas infectivity dropped from 24 to 6% at high relative humidity. While virus on nonporous surfaces can remain infectious for months, the same trends of decreased viability are seen at high humidity (61). Infectious virus is present in scab material (62) and thus a person is considered infectious until all scabs fall off.

Since orthopoxviruses are highly infectious, and it takes only a small number of virions to initiate infection (40), the physical environment occupied by a person with poxvirus disease must be appropriately decontaminated. There are a number of disinfectants that inactivate poxviruses. Using the orthopoxvirus ectromelia virus, White and Fenner examined a range of chemical treatments for surface decontamination (63). For example, a 10-min treatment with 2% phenol or 40% alcohol was sufficient to destroy virus infectivity completely. Sterilization of surfaces can be achieved with formaldehyde gas or a range of commercially available contact sterilants, including fresh solutions of 10% bleach, Spor-Klenz, or Envirocide. Sunlight can also inactivate the virus (64).

EPIDEMIOLOGY

Distribution

Smallpox is no longer known to exist outside of two WHO-designated high containment laboratories.

Monkeypox is now the most frequent human orthopoxvirus causing serious infections (40). Because monkeypox is clinically difficult to distinguish from smallpox, it was not identified as a distinct disease until smallpox was no longer endemic. Monkeypox is found mainly among the inhabitants of small villages in the tropical rain forests of West and central Africa (65), with occasional outbreaks elsewhere (31, 66–68). In 2003 and 2005 there were outbreaks in the United States and Sudan, respectively (69, 70). The US importation was traced to a shipment of animals from Ghana destined for the pet trade (69). Human cowpox is restricted to a region bounded by Norway, northern Russia, Turkmenistan, France, and Great Britain because of the limited ability to remain enzootic except in particular species of rodents. Zoonotic VACV infections are currently confined to Brazil and India, and contact infections from people receiving the smallpox vaccine are limited to countries that vaccinate their military or first responders to infectious disease outbreaks. Some rare human infections have occurred from contact with a recombinant VACV-based rabies vaccine that is used in bait to vaccinate wild animals (71, 72).

Of the remaining human poxvirus infections, ORFV has worldwide distribution. PCPV and BPSV are maintained in dairy herds derived from European herds in all parts of the world. In Great Britain, PCPV infection is enzootic in cattle and, in contrast to CPXV, persists in relatively small herds (73). Tanapox was first observed as an acute febrile illness associated with localized skin lesions, occurring in epidemics in 1957 and 1962 among people living in the flood plain of the Tana River in Kenya (74). It is endemic in this area, in the Democratic Republic of Congo (DRC, former Zaire), and probably elsewhere in tropical Africa (75). Molluscum contagiosum has a worldwide distribution and is very common in certain areas.

Incidence and Prevalence

Subclinical Infection Rate

Subclinical VARV infections among vaccinated subjects were documented in the smallpox eradication program, which, perhaps, is not surprising (2), but their occurrence in naïve subjects is unclear. Studies of human monkeypox in Zaire between 1981 and 1986, after cessation of smallpox vaccination, found that 136 (18%) of 774 unvaccinated contacts of monkeypox patients had serologic evidence of infection; 20% of these persons gave no history of illness, residual skin lesions, or other changes suggestive of monkeypox, and therefore were classified as having subclinical infections (66). More recent studies also provide evidence that subclinical monkeypox infections are likely occurring (76). A serosurvey done in 2003 of 109 children in 3 villages in Ghana who had never received smallpox vaccination and did not report prior monkeypox infection showed that 40 (37%) had antibodies detected against an orthopoxvirus. These villages were studied because the rodents that were imported into the US that caused prairie dog and human monkeypox infections were collected in these locations. Subclinical infections are probably universal for poxviruses, as multiple strains of mice can be infected subclinically with ectromelia virus and act as "silent" reservoirs of infectious virus for contact mice (77).

Patterns of Infection

Variola

Except for VARV and MPXV infections, in which case fatality rates range from 1 to 30%, poxvirus infections of healthy individuals do not result in death.

Transmission of VARV typically occurred within household contacts. For example, an investigation in Pakistan (78) revealed a secondary attach rate of 77% of unvaccinated contacts (and ∽5% in previously vaccinated contacts). From such data, the basic reproductive number (Ro, the number of new cases that one case will generate) for smallpox has been estimated to be between 3 and 10 (79–81). Overall mortality from smallpox was estimated to be 30%. However, in the nonimmune, severe disease and/or death were higher at the extremes of age. For example, in the early 1900s in Liverpool, England, infants <2 years old and unvaccinated adults >40 years old had death rates closer to 50 to 60% (82). In this same study, those previously vaccinated had nearly no death from smallpox. Even those who were vaccinated decades earlier showed death rates at <10%.

Monkeypox

In 1981, the seroprevalence of hemagglutinin-inhibiting (orthopoxvirus) antibodies was 13% of West African children (Ivory Coast and Sierra Leone) and 19% of central African children (DRC) among 10,300 unvaccinated children. A less sensitive but more specific radioimmunoassay adsorption test for MPXV antibodies indicated that 17% of the sera positive for hemagglutinin inhibition contained MPXV-specific antibodies (66, 67). A less specific assay reported similar results, with 1.7% of 994 specimens positive for orthopoxvirus immunoglobulin M in the Likouala region of the DRC (83).

In Africa, over 400 cases of monkeypox were diagnosed between 1970 and 1986, the great majority in parts of Zaire where intensive surveillance supported by the WHO was in operation from 1980 to 1986. The case fatality rate was about 10%. Person-to-person transmission was uncommon but accounted for about 30% of the observed cases. Additional outbreaks were reported in the DRC between 1996 and 1998, but there were concurrent outbreaks of chickenpox, and investigations were hampered by an ongoing civil war (84). In a review by the WHO in 1999, however, it was estimated that about half of the 800 suspected cases in the Kasai Oriental were monkeypox (85). Cases appeared to be no more severe than in the 1980s, but the numbers of index cases and the proportion of secondary cases have increased in recent years (40). From November 2005 to November 2007 (7) surveillance in the DRC identified 760 laboratory confirmed cases in 9 independent health zones, which represented an increase incidence from 0.72 to 14.41 cases/10,000 (∽20-fold increase). Ninety-two percent of cases occurred in people born after mass vaccinations ended in the 1980s. People who were previously vaccinated for smallpox had a 5-fold lower risk of MPXV as compared to unvaccinated (0.78 vs. 4.05 per 10,000), indicating a vaccine efficacy of ∽81% decades after prior vaccination. This increase in human monkeypox infections in the post-vaccination era raises concerns about the emergence of a virus capable of more efficient spread in humans.

In 2003 in the Midwest of the United States, a MPXV infections occurred in prairie dogs housed in a distribution center in close proximity to MPXV-infected African rodents; the prairie dogs acted as an amplifying host for the documented human infections. Based on the case definition at the time, there were 35 confirmed cases and 36 probable cases (86), although other testing techniques identified additional cases (87). A majority of the confirmed infections (19 of 34, 56%) occurred after close contact with MPXV-infected prairie dogs (88), but a number of cases appeared to result from indirect exposure to the virus through fomites or aerosols.

Other Orthopoxviruses

The incidence of other orthopoxvirus infections is far less than that of human monkeypox, with approximately 54 cases of cowpox reported in Europe from 1969 to 1993 (50). Cowpox has been known in Europe for hundreds of years as a disease of cows, manifesting as ulcers on the teats (31). Occasionally, contact with such lesions produced pustular lesions on the hands of milkers. The cow is an accidental and occasional host of CPXV, but cows, cats, and zoo animals can be infected from a rodent reservoir host. Humans may be infected by contact with a wildlife source or with infected animals of several species, especially domestic cats (Fig. 4).

Natural infections with VACV result from epizootics based on virus reservoirs in buffalo and cattle. Buffalopox virus has persisted in India since the cessation of human vaccination (16) and is characterized by pustular lesions on

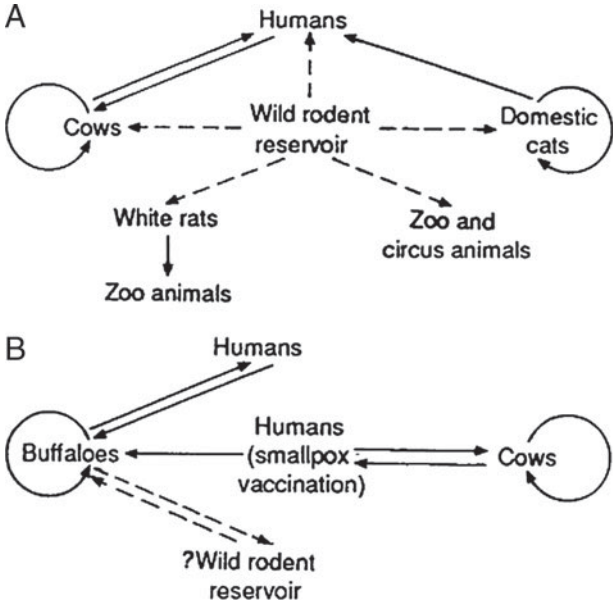

FIGURE 4 Diagram illustrating the epidemiology of cowpox and buffalopox. Solid lines represent known paths of transmission; broken lines represent presumed or possible paths of transmission. (A) Identified wild-rodent reservoir hosts of cowpox include bank voles, wood mice, and short-tailed voles in Great Britain and probably elsewhere in Europe; lemmings in Norway; and susliks and gerbils in Turkmenistan. The traditional liaison hosts from which humans were infected were cows, but currently the most common liaison hosts are domestic cats. White rats, the source of disastrous outbreaks in animals in the Moscow Zoo in 1973 and 1974 (48), probably acquired infection from wild-rodent-contaminated straw or other bedding material. (B) In India, Egypt, and Indonesia, in the days of smallpox vaccination, buffaloes were sometimes infected with vaccinia virus from recently vaccinated humans, causing what was called buffalopox. Buffalopox seems to have disappeared in Indonesia and Egypt but is still a problem in several states of India. Since the cessation of vaccination, infected buffaloes constitute a source of infection of humans. It is possible that the virus can be maintained by serial transmission in buffaloes, but on the analogy of cowpox in Europe, it is possible that there is an unknown rodent reservoir. Reprinted from reference (63) with permission.

the teats and udders of milking buffaloes; occasionally a generalized disease is seen, especially in calves. Sometimes humans in contact with diseased buffaloes are infected (Fig. 4); thus, lesions may occur on the hands and face of milkers. Most strains have restriction maps somewhat different from those of the VACV strains used in India in the days of smallpox vaccination (53). In Brazil, a strain of VACV maintained in cattle is similarly causing zoonotic infections (89).

Recently, a previously unknown orthopoxvirus was identified in a herder in a very rural part of the country of Georgia (21). While only a single case was identified and a field investigation did not show evidence of other people or animals that had been infected with this novel virus, a patient in 2010 in an entirely different part of the country was infected with the same virus.

Parapoxviruses

Human infections with parapoxviruses are an occupational hazard. ORFV, PCPV, and BPSV cause the so-called "barnyard" poxvirus infections, with ORFV infections being the most common. The papules and vesicles on the skin of the lips (scabby mouth) and sometimes around the nostrils and eyes of infected animals serve as a source for human infection. Persistence of the virus in flocks is due in large part to the persistent infectivity of virions in scabs that fall on pasture plants or the soil.

TANV infects both sexes and all age groups and occurs most frequently among persons who work or play close to the Tana River (75). There has been one case of human-to-human transmission reported in the United States (90). The same virus, described variously as Yaba-like disease virus, Yaba-related disease virus, and Oregon "1211" poxvirus, gave rise to epizootics in macaque monkeys in three primate centers in the United States in 1966, in which some of the animal handlers were infected (91). YMTV was first recovered from subcutaneous tumors that occurred in a colony of rhesus (Asian) monkeys in Nigeria (92). Infections of humans with YMTV have not been found in the field in Africa.

Molluscum contagiosum is endemic throughout the world to various degrees. Based on restriction enzyme digests of isolates, there are four subtypes (93). While detailed sequencing of all subtypes has not been done, cross-hybridization revealed that all had similar nucleotide sequences. All subtypes produce similar clinical disease. In the United States, 98% of cases are caused by MCV genotype 1 (25). Molluscum contagiosum is very common in Fiji, where 4.5% of a village population bore lesions (94), and in Papua New Guinea (95). In the Netherlands the cumulative incidence of childhood molluscum contagiosum is about 17% in persons 15 years of age (96). In Australia the seroprevalence was 23% in a representative cross-section of the population (96).

Age-Specific Attack Rates

Information on age-specific clinical attack rates of unvaccinated contacts is available for smallpox, monkeypox, and molluscum contagiosum. For smallpox, while all nonimmune people are susceptible, when smallpox was an endemic infection, the contacts <15 years of age showed the greatest risk of infection; this risk probably reflects the intimacy of physical contact, which is highest among the youngest siblings in a family. For monkeypox in Africa, the primary attack rate reflects the probability of developing the disease after exposure to an animal source. For molluscum contagiosum, studies in New Guinea, Japan, the Netherlands, and the United States found the peak incidence to be in individuals <10 years of age (96, 97).

Risk Factors and High-Risk Groups

Persons of all ages were susceptible to VARV, but the mortality rates were highest in the very young, the elderly, pregnant women, and immunosuppressed individuals. A similar pattern is likely to exist for monkeypox.

Severely immunosuppressed patients have more severe disease with all poxvirus infections, and this has been documented for MCV (98) and ORFV (99). Individuals with atopic dermatitis are probably predisposed to poxvirus diseases of greater severity, especially at the sites of dermatitis. This has been documented for infections with VACV, ORFV, and MCV (96, 100, 101). In the case of molluscum contagiosum, 24% of patients were diagnosed with atopic dermatitis (96). At least two fatal cases of cowpox have been documented in persons suffering from atopic dermatitis (102).

Reported sex differences in smallpox and monkeypox susceptibility more likely reflect risk of exposure and do not represent an intrinsic susceptibility to infection or difference in disease severity. The lack of vaccination immunity is by far the most important risk factor for severe disease in smallpox and monkeypox. The risk of human cowpox increases with ownership of a cat that has access to infected rodent populations. The risk of parapoxvirus infection is occupational.

Reinfections

Recovery from VARV, MPXV, and VACV confers long-lived to lifelong protection from severe disease following reinfection, whereas the generated immunity to CPXV is shorter-lived or is unable to block reinfection with CPXV (3). During the months of an active MCV infection, it is common for the virus to spread to other sites on the skin. However, once all lesions have disappeared, it is unclear if a patient can be reinfected.

Seasonality

In the tropics, smallpox was most prevalent in the cool, dry season. Monkeypox in Africa shows no clear evidence of a seasonal pattern (66).

Transmission

Routes and Risk Factors

Human poxvirus infections can be acquired through multiple routes. VARV is spread by the respiratory route, usually requiring close (household) contact. MPXV infection from the zoonotic host probably occurs through puncture wounds or microbreaks in the skin. MPXV human-to-human transmission is likely to occur via the respiratory route or through mucosal surfaces (65). CPXV and parapoxvirus infections are the consequence of the introduction of virus either directly or indirectly into breaks in the skin. MCV is spread by fomites or close contact among children and can be sexually transmitted among adults.

The transmission risks of MPXV infection are multifactorial. Primary MPXV infection is associated with adult male activities such as hunting and carcass preparation, whereas female caregivers are at greater risk for human-to-human transmission.

Parapoxvirus infections occur through occupational exposure. For example, marine mammal technicians are at risk for sealpox through bites from seals or sea lions (103).

Sexual activity is a risk factor for molluscum contagiosum in adults. Genital molluscum has become a much more common infection in the last two decades and can be an epidemiological marker for sexually transmitted diseases. The disease can be a troublesome complication of AIDS in Western countries, especially in homosexual men (104). For children, there is an association between swimming pools and MCV (97).

Nosocomial Infection

VARV and MPXV, which can be transmitted by respiratory droplets, can cause nosocomial infections. In 1970, a smallpox patient maintained in an isolation ward in a general hospital in Meschede, Germany, infected 17 patients that were in rooms that were one to two floors above the index patient l (105). While face-to-face contact is the most common route of spread, it was thought that the spread in the hospital occurred because the index patient had a severe persistent cough and that strong air currents and low humidity in the hospital allowed airborne transmission. In 2003, extended interhuman transmission of MPXV was documented on the grounds of a community hospital in the DRC (106).

Duration of Infectiousness

The ability of human poxvirus infections to transmit infectious virus is dependent on the generation of a lesion in the cornified or mucosal epithelium that ulcerates, releasing virus into the environment. For VACV, CPXV, parapoxviruses, and MCV, the lesions are usually in the cornified epithelium and are likely infectious until scab formation or reabsorption. For smallpox and probably monkeypox acquired from human index cases, the mucosal lesions of the respiratory tract are a greater source of infectious virus than lesions of the cornified skin and are more important in the natural transmission cycle of the virus. VARV is detected 2 to 4 days following the onset of fever and reaches the highest level on the third and fourth days of disease (i.e., just after rash appearance) in oropharyngeal secretions of smallpox patients. The level of virus was greatest and persisted the longest in cases that had confluent ordinary-type (lesions on face or extremities are confluent) or flat-type smallpox. High levels of virus in secretions correlated with infectiousness of the patient, a period that usually lasted for the first week of the rash.

PATHOGENESIS IN HUMANS

Incubation Period

The incubation period for human poxvirus infections has been thoroughly documented only for smallpox, for which it was 10 to 14 days (2). A limited number of case studies of human-to-human transmission of MPXV suggest a similar incubation period (66).

Patterns of Virus Replication and Spread

Our understanding of the pathogenesis of smallpox is based on animal models: namely, ectromelia virus in mice [(107, 108); Fig. 5] and MPXV in monkeys (109). Based on these models and epidemiologic observations of smallpox, infection with VARV usually occurs by the inhalation of virus released from oropharyngeal lesions into the saliva and respiratory secretions during the first week of the rash. After infection of cells in the upper and lower respiratory tract of contacts, macrophages became infected and entered the lymphatics. Infected white blood cells sometimes entered

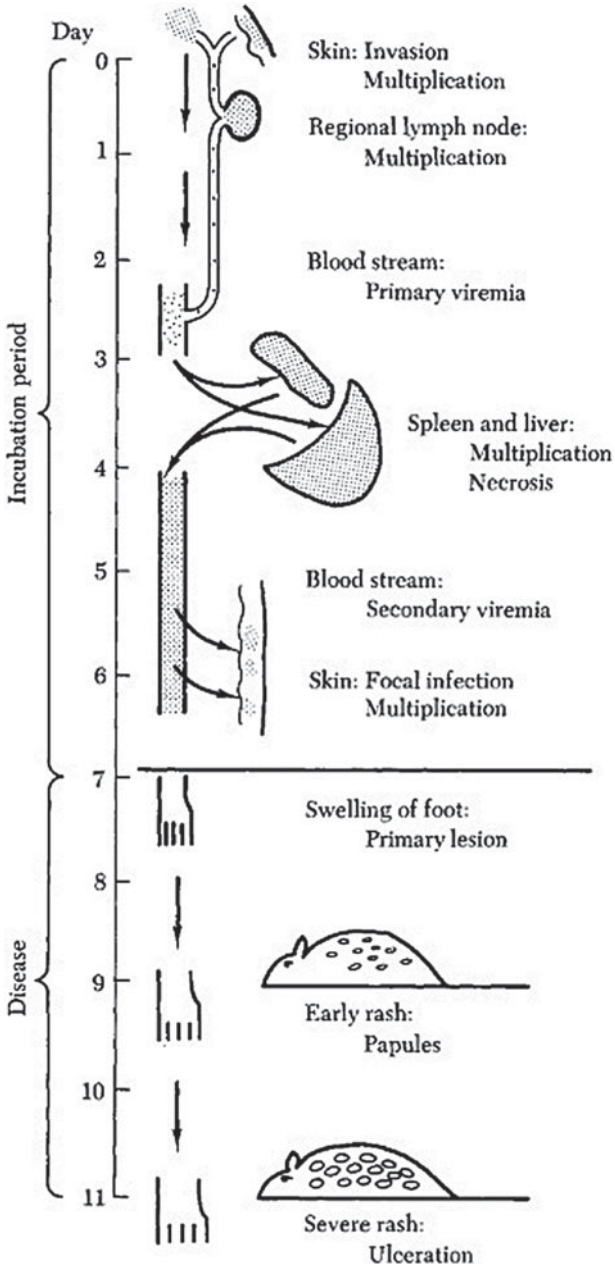

FIGURE 5 Diagrammatic representation of the mode of spread of ectromelia virus through the body in mousepox. Reprinted from reference (107) with permission.

the bloodstream at this stage, or in any case by about the fourth day. Lymphoid tissue and internal organs were infected following this primary viremia. The initial stages of infection produced neither symptoms nor a local lesion, and patients were not infectious during the incubation period. Approximately 8 to 10 days following infection, virus produced from infected internal organs (secondary viremia) localized in small vessels of the dermis and led to infection of the underlying dermis of the mucosal and cornified epithelium. The rash was detected approximately 10 days after infection, evolved through a number of distinct stages, and resolved with scab separation ∽32 days following infection. Virus was difficult to detect in blood from cases of ordinary-type

TABLE 3 Complications of smallpox vaccination in the United States in 1968

Status and age	No. of vaccinations	Complication rate (cases per million)				
		Postvaccinial encephalitis	Progressive vaccinia	Eczema vaccinatum	Generalized vaccinia	Other[a]
National survey[b]						
Primary vaccination						
<1 yr	614,000	6.5	0	8.1	70.0	27.7
1–4 yr	2,733,000	2.2	0.4	11.3	17.2	47.9
>5 yr	2,247,000	2.7	1.8	9.3	16.9	23.6
Revaccination						
<1 yr	0					
1–4 yr	478,000	0	0	2.1	0	4.2
>4 yr	8,096,000	0	0.7	0.9	1.2	1.7
Ten-state survey[c]						
Primary vaccination						
<1 yr	71,000	42	0	14	394	1,099
1–4 yr	317,000	9.5	3.2	44	233	972
>4 yr	262,000	7.6	0	34	149	748
Revaccination						
<1 yr	0					
1–4 yr	55,000	0	0	0	0	200
>4 yr	943,000	2.1	3.2	3.2	9.5	81

[a]Includes accidental infection, erythema multiforme, bacterial infections, and severe "normal" reactions requiring medical care.
[b]Data from reference (138).
[c]Data from reference (139).

smallpox at all times during disease; however, this was not the case with hemorrhagic-type smallpox, in which high titers were readily detectable in blood of all cases. The outcome of the infection was either death, which was said to be usually due to "toxemia," or recovery, with complete elimination of the virus but sometimes with sequelae. The most common sequelae were pockmarks, which could occur all over the body but were usually most profuse on the face (110). Blindness was an important but rare complication. Recovery was accompanied by prolonged immunity to reinfection.

The pathogenesis of monkeypox probably follows at least two distinct courses dependent on the route of transmission. Inhalation of MPXV, which is likely the mechanism of infection between an index case and contacts, has a pathogenesis very similar to that of the smallpox virus. Infections from an animal source may occur through additional exposure routes including puncture wounds or small lesions on the skin or the oropharynx. Infections through the skin likely have a distinct pattern of pathogenesis compared to that following a respiratory infection. This supposition is based on the fact that the smallpox case fatality rate was 20 to 30% following inhalation but was dramatically lower, at 0.5 to 2%, if virus was introduced into the cornified epithelium (variolation; see below).

Except for VARV and MPXV, poxvirus infections of humans are most often through the cornified epithelium. The introduction of CPXV, VACV, or parapoxviruses into the skin causes localized distinct pustular lesions at the site of introduction that have species-specific distinctions. In immunocompetent individuals, systemic infections occur at a low frequency following VACV infection (Table 3, generalized vaccinia) and are extremely rare for CPXV or other human zoonotic poxvirus infections. The primary lesions heal over in 3 to 6 weeks following infection.

Disease Production and Pathologic Features

The secondary skin lesions of smallpox develop through a series of characteristic stages that presumably begin with a virus-infected cell entering into the dermis through diapedesis, which is followed by local replication. The focus of infection becomes mildly inflamed due to the local release of cytokines and chemokines, which attract circulating inflammatory cells. Virus replication spreads from the dermis into the epidermis. Cells in the epidermis become swollen and vacuolated and stain for the characteristic B-type (Guarnieri) inclusion bodies (Fig. 6). The rupturing cells contribute to early vesicle formation. Lesions evolve from macules to papules and into pustules through the migration of polymorphonuclear leukocytes from the subpapillary vessels into the epidermal lesion. The pustule umbilicates, and with the development of an effective immune response,

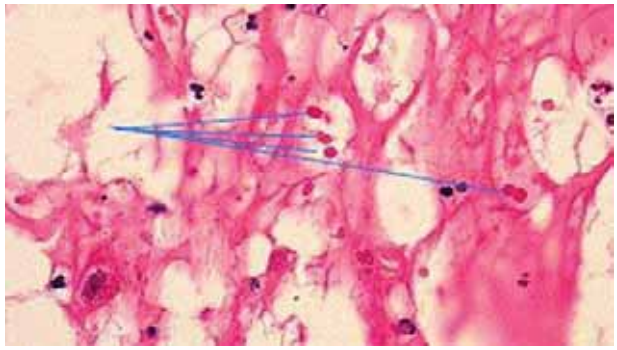

FIGURE 6 Intracellular inclusions (Guarnieri bodies) in a biopsy from a patient from Somalia prior to smallpox eradication. Courtesy of Dr. Dirk Elston.

the healing process commences and results in regeneration of the epidermis and the start of scab formation at ~16 days postinfection. Autopsies of people who died from smallpox showed enlarged and engorged organs. Histology showed ballooning of endothelial cells and an inflammatory infiltrate that was mainly mononuclear cells (111). High titers of virus can be found in internal organs, including liver, spleen, lymph nodes, and bone marrow (2).

The earliest histologic changes observed in VACV lesions following vaccination are cytoplasmic and perinuclear vacuolation in the epithelium, which are accompanied by coagulation necrosis, intercellular edema, and vesicle formation (2). By 48 h postvaccination, a cup-shaped vesicle traversed by an eosinophilic reticular network appears with the roof and floor formed by the stratum corneum and dermis, respectively. The region is characterized by edema, free erythrocytes, and a progressive infiltration of mononuclear and polymorphonuclear cells, which with time formed a dense, homogeneous, deeply staining reticulum that formed the crust beneath which epithelial outgrowth occurred.

In contrast to lesions of smallpox and monkeypox, parapoxvirus lesions are markedly proliferative (112). Changes that occur early in the proliferating keratinocytes include nucleolar enlargement and focal lysis of keratin fibrils. Extreme swelling of the cells results in ballooning degeneration, which, when accompanied by B-type cytoplasmic inclusion bodies and nuclear shrinkage, is pathognomonic of ORFV lesions. Dermal infiltration with monocytes and lymphoid cells is prominent around hyperemic capillaries and venules, and infection of the endothelial cells may produce endothelial proliferation.

Tanapox lesions histologically show some typical features of an orthopoxvirus skin lesion, i.e., marked thickening of the epidermis with extensive ballooning degeneration of the prickle cell layer. The cytoplasm of swollen epidermal cells is filled with large, pleomorphic, granular, eosinophilic, B-type inclusion bodies. YMTV produces tumors in monkeys and humans after subcutaneous or intradermal inoculation. These are composed of masses of histiocytes, which are later infiltrated with lymphocytes and polymorphonuclear cells. No true neoplastic proliferation occurs, and the lesions regress as the immune response develops (113).

The typical molluscum contagiosum lesion consists of a localized mass of hypertrophied and hyperplastic epidermis extending down into the underlying dermis and projecting above the adjacent skin as a visible tumor. Each infected keratinocyte is many times larger than normal, and the cytoplasm is filled with a large hyaline acidophilic granular mass known as the molluscum body (Fig. 7), which pushes the nucleus to the edge of the cell. The core of the lesion consists of degenerating epidermal cells with inclusion bodies and keratin, which uninfected cells continue to produce. Unlike with orthopoxvirus lesions, there is no vesicle formation or inflammatory reaction unless secondary bacterial infection has occurred. The lack of inflammation is due to the restricted replication of the virus to the keratinocyte of the epidermis, the release of little antigenic material or virus from infected cells, and the expression of immunosuppressive proteins (36).

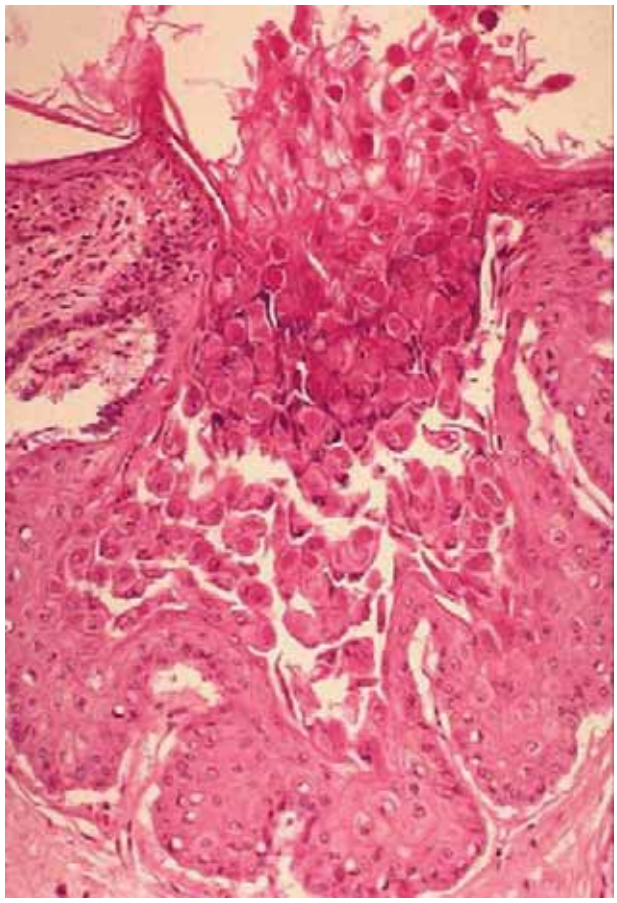

FIGURE 7 Section of a skin lesion of molluscum contagiosum. Courtesy of CDC/ Dr. Edwin P. Ewing, Jr., Public Image Library ID# 860.

Immune Responses

Innate Responses

Innate responses have not been studied in human poxvirus infections but can be inferred from our knowledge of experimental mousepox (108). Infection with necrosis initiates a cascade of cytokines and chemokines and cellular activation. The cascade is further amplified by the autocrine and paracrine proinflammatory activities of the resident and infiltrating cells (monocytes, granulocytes, and natural killer cells) as well as the vascular endothelial cells. In MCV this process is absent or transient in nature.

Adaptive Immunity

The adaptive response is characterized by the production of cytotoxic and cytokine-secreting T cells and antibody-secreting B cells. The cytotoxic T cells kill infected cells prior to the completion of the virus replication cycle by recognizing viral peptides in the context of antigens from the HLA complex. In animal models the humoral response plays a major role along with T cells in recovery from primary infection and is critical for protection from reinfection (114–116). The most important targets of neutralizing antibodies are unique proteins found on the surface of mature virus and extracellular virus (117).

The 2003 importation of animals from Africa infected with monkeypox virus provided an opportunity to study the immune response in infected humans. Antibody responses (IgG and IgM) could be detected within the first week after rash onset, often within the first day or two (118). T-cell responses were measured in people infected with monkeypox (87, 119), but samples from acutely infected patients were

not obtained. The severity of illness did not appear to be associated with the magnitude of the T-cell response (119).

The acute viral infections with VARV, CPXV, and VACV produce lasting long-term immunity. The more chronic infection by MCV initially provokes little immunity, and in individual patients the lesions may persist for as little as 2 weeks or as long as 2 years, without any sign of inflammation. The lesions are noteworthy for the absence of reactive cells. Virus-specific antibodies are demonstrable in about 70% of patients (120).

Correlates of Disease Resolution and Protection

In human poxvirus infections, scab separation of the last active lesion indicates disease resolution. In smallpox, this separation occurred at ∽32 days following infection. The presence of scars from VARV, MPXV, or VACV lesions is considered a correlate of immune protection.

CLINICAL MANIFESTATIONS

Major Clinical Syndromes

Variola and Monkeypox

The prodrome of smallpox or classic monkeypox includes a spiking fever, malaise, and possibly headache followed by the development of a rash (2, 66). There are two clinical types of smallpox due to two viral variants, variola major and variola minor (alastrim) (2). Variola major had an overall case fatality rate of 20 to 30% in unvaccinated persons with mortality rates much higher at the extremes of age. Variola minor had a case fatality rate of about 1%. The onset was acute, with fever, malaise, headache, and backache. Three or four days after the onset of symptoms the characteristic rash appeared, first on the buccal and pharyngeal mucosa and then on the face, forearms, and hands. The lesions began as macules, which soon became firm papules and then vesicles, which quickly became opaque and pustular. About 8 or 9 days after the onset, the pustules became umbilicated and dried up. The distribution of the rash, as well as its evolution, was highly characteristic, being usually most profuse on the face and more abundant on the forearms than the upper arms and the lower legs than the thighs, and relatively sparse on the trunk, especially the abdomen (Fig. 8, left panel).

The clinical features of monkeypox are indistinguishable from those of ordinary-type smallpox, except that enlargement of cervical, and often the inguinal, lymph nodes is much more pronounced than in smallpox (Fig. 8, middle and right panels). The evolution of the rash in monkeypox in central Africa is similar to that of smallpox. The rash is most severe on the extremities, and the lesions evolve as a group. This is in contrast to the disease observed in the 2003 US outbreak, in which distinct differences were noted in the morphology, evolution, and absolute numbers of skin lesions. In the US outbreak the lesion morphology varied from case to case and even within a family, and the lesions healed with a distinctive and prominent hemorrhagic crust (122).

Although like smallpox, monkeypox is clinically distinct from chickenpox, misdiagnosis of monkeypox for chickenpox and chickenpox for monkeypox is quite common (123). In general, Africans may be more susceptible to severe disease due to poor nutrition or coinfections with other pathogens. Two reports have documented coinfections of MPXV and varicella-zoster virus, with one fatal outcome (84, 124). AIDS increases the morbidity and mortality of humans when they are concurrently infected with *Mycobacterium tuberculosis* (125, 126), and a similar effect would be expected following coinfection with MPXV.

At least two distinct clades of MPXV exist, one found in West Africa and the other in central Africa (122, 127). Epidemiological studies and experimental inoculation of nonhuman primates suggest that the West African isolates

FIGURE 8 Clinical features of smallpox and monkeypox. (Left) The rash of smallpox in a boy in Bangladesh in 1974.[a] (Middle) Rear view of a 7-year-old girl from Zaire with monkeypox, on the eighth day after the appearance of the rash. Note the enlargement of the cervical lymph nodes, a feature not seen in smallpox.[b] (Right) Another example of lymphadenopathy associated with monkeypox infection.[c]
[a]Courtesy of CDC/Jean Roy, Public Image Library ID# 10654.
[b]Reprinted from reference (121) with permission.
[c]Courtesy of CDC/Brian W. J. Mahy, Public Image Library #12778.

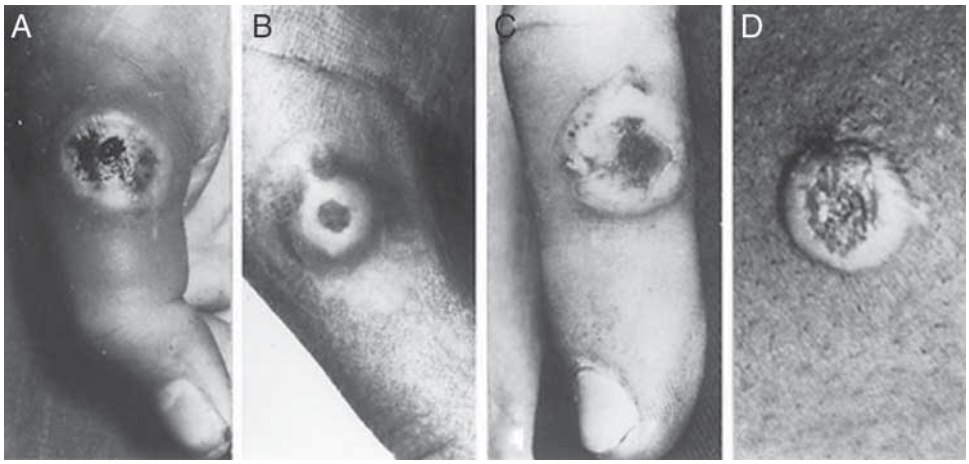

FIGURE 9 Localized zoonotic infections with poxviruses. (A and B) Lesions on hands acquired by milking infected cows: cowpox, caused by an orthopoxvirus (A), and milker's nodes, caused by a parapoxvirus, PCPV (B). (C) Parapoxvirus lesion of ORFV, acquired by handling sheep or goats suffering from contagious pustular dermatitis. (D) Lesion of tanapox on an arm of a child in the DRC. The virus was transmitted mechanically by mosquitoes from an animal reservoir host.
Panels A and B courtesy of D. Baxby; panel C courtesy of J. Nagington; panel D courtesy of Z. Jezek.

are less virulent than those from central Africa. The lesion character in the 2003 US outbreak was distinct from that of classic African monkeypox, and infected patients had, on average, fewer lesions (122). Patients also presented with a broad range of nonspecific signs and symptoms, with and without rash (88, 128). Interestingly, no deaths were reported, perhaps due to the fact that the virus was the less virulent West African strain (69).

Vaccinia and Cowpox

The prodrome of cowpox may include fever and myalgia but with less severity compared to that of smallpox and monkeypox. CPXV lesions are often found on the thumbs, the first interdigital cleft, and the forefinger. The lesions are quite distinctive (Fig. 9A); CPXV causes pustular lesions like those of VACV, and the parapoxviruses produce nonulcerating nodules. The CPXV lesions pass through stages of vesicle and pustule before a deep-seated, hard black eschar forms 2 weeks later. Cowpox was traditionally associated with the milking of cows, but many cases involve no such history, and domestic cats now constitute a more important source of human infection.

In the first 6 days following vaccination with VACV (Dryvax), fever, headache, muscle aches, chills, nausea, and fatigue are reported in 3%, 44%, 39%, 13.5%, 19%, and 53% of 665 subjects, respectively (129). The current smallpox vaccine (ACAM 2000) has similar clinical findings (130). In tanapox the first signs of infection are mild fever, headache, and myalgia. The primary response to VACV vaccination evolves through a characteristic series of lesions (Fig. 10). VACV infection via vaccination causes a papule to appear at the vaccination site 4 to 5 days after vaccination; 2 to 3 days later, this becomes vesicular and constitutes the umbilicated and multiocular "Jennerian vesicle." The contents rapidly become turbid because of the infiltration of inflammatory cells with the central lesion surrounded by erythema and induration, which reaches maximum diameter on the 9th or 10th day. At this time the draining lymph nodes in the axilla are enlarged and tender, and many patients have mild fever. The pustule dries from the center outward, and the brown scab falls off after about 3 weeks, leaving a scar by which previous vaccination can be recognized for many years.

Parapox Viruses

There are few, if any, signs or symptoms of parapoxvirus infection except occasional fever before development of the primary lesion. Orf and milker's nodules are acquired by contact with infected sheep and cows, respectively, and the localized lesions are usually found on the hands. The lesions of orf are rather large nodules that may be multiple, and the surrounding skin is inflamed (Fig. 9C). Orf lesions are rather

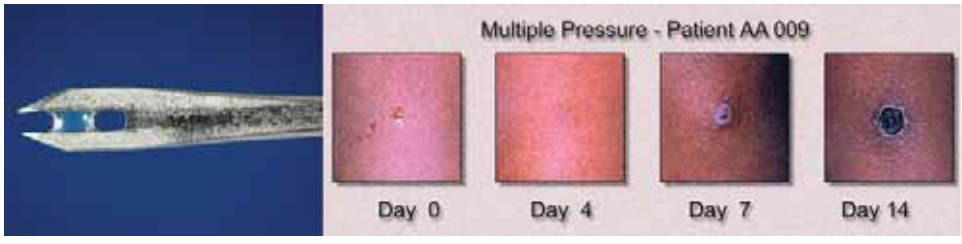

FIGURE 10 Smallpox vaccination. (Left) Tip of a bifurcated needle used to vaccinate individuals containing some vaccine solution.[a] (Right) Primary response to vaccination; typical vesiculo-pustular response.[b]
[a]Courtesy of CDC, Public Image Library ID# 2667.
[b]Courtesy of CDC/Arthur E. Kaye, Public Image Library ID# 3238.

FIGURE 11 Molluscum contagiosum lesions. (Left) 9-year-old boy with multiple skin-colored molluscum papules on his face and neck. Reprinted from reference (136) with permission. (Right) Molluscum in a 33-year-old man with AIDS. Reprinted from reference (137) with permission.

painful and may be accompanied by low-grade fever and swelling of the draining lymph nodes. The lesions of milker's nodule (Fig. 9B) are hemispherical, highly vascular papules that appear 5 to 7 days after exposure and gradually enlarge into purple nodules up to 2 cm in diameter. They are relatively painless but may itch, and they do not ulcerate. The granulation tissue that makes up the mass of the nodule gradually becomes absorbed, and the lesions disappear after 4 to 6 weeks. The only evidence of systemic infection is occasional slight swelling of the draining lymph nodes. Human infections with BPSV are less common than with the other two parapoxviruses that infect humans, probably because contact between animal handlers and lesions of BPSV are less common than those of shearers with orf and milkers with milker's nodules (131, 132). The BPSV lesions appear as circumscribed wart-like nodules that gradually enlarge until they are 3 to 8 mm in diameter.

The first symptoms following a TANV infection are a mild preeruptive fever, sometimes accompanied by severe headache, backache, and myalgia, often with itching at the site where the skin lesion develops (75, 133, 134). Two to four days following onset of fever, a usually solitary erythematous macule appears on the skin. Initially there is a small nodule, which soon becomes papular and reaches a maximum diameter of about 15 mm by the end of the second week. The appearance of the skin lesion of tanapox is illustrated in Fig. 9D. The nodule is surrounded by an edematous zone and a large erythematous areola. The draining lymph nodes are enlarged and tender from about the fifth day. The skin lesion usually ulcerates during the third week and then gradually heals within 5 to 6 weeks, leaving a scar. In Kenya, the lesions are almost always solitary and on the upper arm, face, neck, and trunk (74), but in the DRC, 22% of patients have had multiple lesions (75). YMTV produces large protuberant tumors in monkeys, which are histiocytomas containing poxvirus particles (113, 135). Rarely, similar lesions may be produced in humans by accidental contact with infected monkeys or after inoculation.

Molluscum contagiosum

Molluscum contagiosum is characterized by multiple, small, noninflamed nodules in the skin scattered over the body or, in adults, usually in the thighs, genital or anal region (25). The lesions of molluscum contagiosum are pearly, flesh-colored, raised, umbilicated nodules in the epidermal layer of the skin, usually 2 to 5 mm in diameter (Fig. 11, left). Rarely, lesions will present as a large lesion called giant molluscum (>5 mm in diameter). The incubation period varies from 14 to 50 days. The nodules are painless, and at the top of each there is often an opening through which a small white core can be seen. The lesions often persist for months but ultimately resolve spontaneously or following trauma or bacterial infection. More severe disease can be seen in immunocompromised hosts (Fig. 11, right).

Age-Related Differences

In smallpox and monkeypox the young were more likely to be infected than the old, and the disease was most severe in the young and old. The exception was the 2003 US monkeypox outbreak, in which the majority of the cases were in adults (88); however, the pediatric patients were more likely to be hospitalized in an intensive care unit. Cowpox in children was usually more severe than vaccination with vaccinia virus (47, 50).

Complications

Smallpox

Three other clinical presentations of smallpox were recognized. The rare hemorrhagic-type smallpox, most common in pregnant women, was associated with petechiae in the skin and bleeding from the conjunctiva and mucous membranes, very severe toxemia, and early death. Flat-type smallpox was characterized by intense toxemia and slow evolution of the skin lesions, which were usually flat and soft; most such cases were fatal. Modified-type smallpox was seen in persons who had been vaccinated, usually many years earlier; the disease was mild and the skin lesions evolved quickly and were often sparse.

Vaccinia

Smallpox immunizations with VACV results in occasional serious complications (Fig. 12; Table 3) (138–140). Vaccination against smallpox is undoubtedly associated with both increased reactogenicity and more complications than any other of the commonly used vaccines (141). Progressive vaccinia (Fig. 12B) occurs only in persons with deficiencies of the cell-mediated immune system. Vaccinated subjects with agammaglobulinemia but a normal cell-mediated response recover normally (142, 143). Eczema vaccinatum (Fig. 12A) occurs in persons with eczema or atopic dermatitis, which affects over 10% of the United States population. However, not every person with this skin condition develops eczema vaccinatum and thus it is not known what

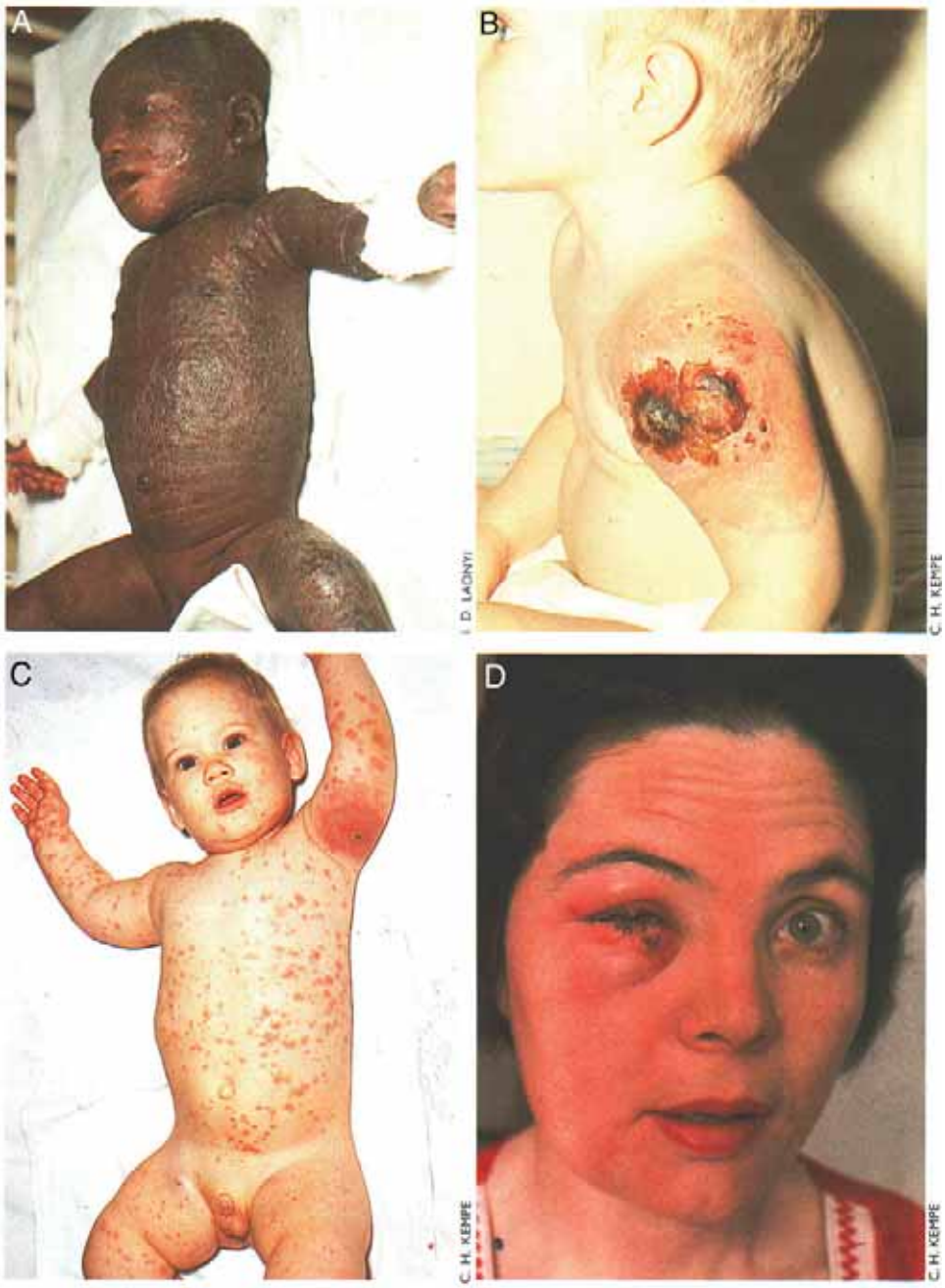

FIGURE 12 Severe complications of vaccination. (A) Eczema vaccinatum in an unvaccinated sibling of a vaccinated individual. (B) Progressive vaccinia (vaccinia gangrenosum), which was fatal in a child with a congenital defect in cell-mediated immunity. (C) Generalized vaccinia, 10 days after primary vaccination; benign course, no scarring. (D) Ocular vaccinia after autoinoculation. Reprinted from reference (2) with permission.

factors are involved in those who develop this severe complication (144). In those who do develop this complication, it can be life threatening if recognized late in the course of disease (145). Treatment with VIG (and other experimental anti-poxvirus agents) can lead to viral control and clearing of the lesions. Because vaccinia replicates preferentially in immature keratinocytes, other inflammatory dermatologic conditions or cutaneous injury sites can be accidentally infected by VACV. Generalized vaccinia comprises a generalized vaccinial rash, sometimes covering the whole body (Fig. 12C) and occurring 6 to 9 days after vaccination. It is not associated with immunodeficiency, and the prognosis is good. Postvaccinial encephalitis is an unpredictable complication (146). In children less than 2 years old there is very occasionally a general encephalopathy associated with demonstrable viremia that occurs 6 to 10 days after immunization and is characterized often by convulsion, hemiplegia, and aphasia (147). In subjects older than 2 years, the complication occurs about 11 to 15 days after immunization. The onset of disease is marked by fever, vomiting, and other

symptoms of cerebral involvement. The pathological findings resemble those of other postinfectious encephalitides, and the case fatality rate is approximately 45% (146). Perivenular inflammation and demyelination are the principal lesions. Accidental infection of some part of the body distant from the inoculation site is the most common complication. From 1963 to 1968, ocular vaccinia (Fig. 12D) was found to have occurred in 348 persons (including 259 vaccinees and 66 contacts) (148). The cornea was involved in 22 of these cases, and 11 persons had residual visual defects.

Clinical Diagnosis

When smallpox was endemic, the diagnosis of most cases could be made clinically according to the distribution and evolution of the rash. In regions where disease was not expected, modified-type variola major and variola minor were often confused with chickenpox, but the evolution and distribution of the rash in the two diseases were usually distinctive. In smallpox, lesions developed simultaneously all over the body, most obviously on the face and limbs rather than the trunk, whereas, the skin lesions of chickenpox were more superficial and appeared in "crops," more apparent on the trunk than on the face and extremities.

The diagnosis of zoonotic poxvirus infections facilitated by the geographic location and epidemiological features of cases can be of help in the differential diagnosis, unless the disease appears in a nonenzootic region, as in the case of importation of MPXV into the United States in 2003. Like smallpox, monkeypox can be misdiagnosed as chickenpox. Poxvirus infections acquired from contact with cows must be differentiated from among CPXV, PCPV, and BPSV. If the infection is acquired from sheep, it is likely ORFV. Tanapox is known to occur in animal attendants in contact with monkeys infected with TANV or YMTV or wild animals in regions of Africa where these viruses are endemic.

Diagnosis of a VACV infection can be problematic in subjects infected by contact with a vaccinee. The history of contact with a recently vaccinated person or with a laboratory source of VACV, or with a buffalo with buffalopox or cattle infected with cantagalo virus, should arouse suspicion; the diagnosis can be confirmed by recovery and characterization of the virus.

Molluscum contagiosum is endemic in most human populations. The diagnosis can be made clinically from the appearance of the lesions and their chronic nature. Occasionally, solitary lesions on the face or neck may be misdiagnosed as basal cell carcinoma. Cutaneous cryptococcosis can mimic molluscum lesions in patients with AIDS.

LABORATORY DIAGNOSIS

Historically, the major diagnostic criteria for poxvirus infections are the size and morphology of the poxvirus virion in negatively stained preparations viewed with an electron microscope (Fig. 1). This approach is giving way to detection by PCR.

Virus Isolation

Specimen Types

Specimens are usually fluid from primary or secondary vesicles or scabs (or corneal swabs), all of which contain sufficient viral material to be used for PCR identification.

Cell Culture

Historically, species diagnosis was made by observation of the characteristic pocks on the chorioallantoic membrane of the developing chicken embryo. In combination with growth ceiling temperature, the careful diagnostic technician could use the observation of pocks to differentiate CPXV, VACV, MPXV, and VARV. In more recent times, cell culture in combination with antigen staining has been used as a confirmatory assay for electron microscopy. Orthopoxviruses produce cytopathic effect in Vero cells. Parapoxviruses from human lesions grow in primary bovine or ovine embryo kidney or testis cells or in primary human amnion cells. Once isolated, they grow well in human embryonic fibroblasts and LLC-MK2 cells. Yatapoxviruses grow in human thyroid cells, vervet monkey and patas monkey kidney cells, and WI-38, HEp-2, and Vero cells, producing focal lesions characterized by intense granularity followed by rounding up of the cells (74).

Antigen Detection

Historically, antigen detection protocols rarely could differentiate poxviruses at the species level. Thus, VACV could not be discriminated from MPXV or VARV. This approach is rarely used since the development of PCR.

Histology

Molluscum contagiosum can be diagnosed solely on clinical grounds based on the gross appearance of lesions and their chronic nature; however, giant molluscum can be confused with other disorders. The differential diagnosis is readily confirmed by hematoxylin and eosin staining of biopsy material, and the presence of "molluscum" or Henderson-Patterson bodies is pathognomonic for disease. Electron microscopy and PCR can also be used for diagnosis, but these are usually not warranted (149).

Nucleic Acid Detection

The requirement for specific banks of tissue culture cells for poxvirus isolation and the long time to generate assay endpoints spurred the development of the PCR assay as the mainstay diagnostic method. PCR has become the method of choice to distinguish 11 species of *Orthopoxvirus* from each other and variola major virus from variola minor virus (30), four species of *Parapoxvirus* (150), MCV (149), and three species of *Yatapoxvirus* (134, 151). Testing for poxviruses is not routinely done by clinical microbiology labs, and thus testing is done through local public health labs in conjunction with the CDC.

Serologic Assays

Presently, there are no sensitive, specific, and reliable serologic assays that retrospectively differentiate among orthopoxvirus infections. Also, following ORFV, PCPV, and MCV infections, antibodies have not always been detected or persist only transiently. An immunoglobulin M-capture enzyme-linked immunosorbent assay (ELISA) using VACV antigen has been developed as an epidemiological tool; however, this assay cannot directly differentiate among antibodies induced by orthopoxvirus species (118). The lack of a single species-specific ELISA for orthopoxvirus infections can be overcome by the use of an ELISA based on antigens of the orthopoxvirus species that need to be differentiated and comparison of titers in serum using these antigens along with known serologic standards (87). For example, in the 2003 monkeypox outbreak, the serologic assay needed to discriminate between an acute MPXV infection, residual immunity from smallpox vaccination in an uninfected subject, and MPXV infection of an individual with residual vaccine immunity.

PREVENTION

General

Environmental surroundings can be contaminated from lesional sources of poxvirus. For VARV and possibly other orthopoxviruses, this contamination is not usually of epidemiological significance, as the virions are locked in inspissated, pustular fluid that is difficult to resuspend in the air in a respirable form. In contrast, transmission of MCV and parapoxvirus infections have been documented with a large number of fomites (22, 152). Thus, environmental surfaces potentially exposed to poxviruses must be thoroughly disinfected. Of the human poxvirus infections, only adults with MCV have a behavioral component. The majority of adult MCV infections are the result of close skin-to-skin contact with an infected site that occurs during interactions such as sexual intercourse (153).

Isolation of Infected Persons

Historically, the quarantine of communities and the isolation of infected individuals or their contacts were implemented for smallpox, and, to a certain extent, for human monkeypox because the infection can be transmitted by respiratory droplets. For those with smallpox disease, isolation was for 2 to 3 weeks, which represented the time for lesions to heal and scabs to fall off. Contacts of a case of smallpox would be quarantined for 2 to 3 weeks, which represented the asymptomatic incubation period. Vaccinees are counseled to avoid contact with the extremely young (<1 year old), with pregnant women, and with persons with atopic dermatitis or on immunosuppression.

Passive Immunoprophylaxis

Historically, intramuscular administration of vaccinia immunoglobulin (VIG), a product derived from the pooled plasma of vaccinated individuals, was used to treat progressive vaccinia (vaccinia necrosum), eczema vaccinatum, and certain autoinoculations, although efficacy has not been demonstrated through controlled clinical trials (154). VIG was reported to halt formation of new lesions and to cause rapid clinical improvement in cases of generalized vaccinia and eczema vaccinatum (110). One large study suggested that postexposure treatment of contacts of patients with smallpox with vaccination and VIG appeared to be more efficacious than vaccination alone. Smallpox developed in 5 of 326 contacts who received VIG, compared to 21 of 379 controls, for a relative efficacy of 70% in preventing smallpox (155, 156). In 2005, the Food and Drug Administration (FDA) approved new stocks of VIG by DynPort Vaccine Company, LLC for intravenous use (VIG-IV). It is used at a dose of 6,000 units/kg to treat complications of vaccinia virus vaccination. Repeat dosing has been used in severe cases. VIG is part of the US Strategic National Stockpile, and its use should be performed with the help of CDC's Smallpox Vaccine Adverse Events Clinical Consultation Team (phone: 404-639-3670). Prior use of VIG to treat smallpox did not always provide reproducible and clear-cut benefit. A highly potent cocktail of monoclonal antibodies generated against key neutralizing epitopes on surface proteins of intracellular mature virus and extracellular enveloped virus has shown promise in nonhuman primate and mouse models (157, 158).

Active Immunization

Vaccination has a long history for the prevention of poxvirus infections. As early as the 10th century in Asia, the inoculation of pustule fluid or scab material into the skin ("inoculation" or "variolation") was shown to protect against "natural" smallpox, with case fatality rates of 0.5 to 2% instead of 20 to 30%. Variolation was introduced into Europe early in the 18th century, and in some countries, notably England, it was practiced on a large scale.

In 1796 Edward Jenner showed that persons who had been inoculated with a related orthopoxvirus, CPXV, were completely resistant to smallpox (3). Vaccination (named from *vaca*, Spanish for "cow") led to a great decline in the incidence of smallpox in many countries. By the early 1950s many of the industrialized countries had eliminated endemic smallpox, and in 1958 the World Health Assembly accepted the concept of global eradication of smallpox. Realization of this goal faltered for several years, but in 1967 a Smallpox Eradication Unit was established at WHO headquarters, and the goal of global eradication within a decade was pursued with vigor and enthusiasm. Helped by the availability of adequate amounts of potent and stable freeze-dried vaccine, a change in strategy from reliance on mass vaccination alone to ring vaccination supported by surveillance and containment, and a new and simple inoculation device (the bifurcated needle, see Figure 10), the WHO program achieved its target in October 1977, and the world was certified free of smallpox by the World Health Assembly in May 1980 (2, 159).

The traditional smallpox vaccine could be given as late as 4 days postinfection (160) and still modify the disease course. When the vaccine was administered optimally, nearly complete protection against smallpox was maintained for about 5 years, and various degrees of protection were maintained thereafter (88% at 10 years and 50% at 20 years after vaccination). This vaccine was estimated to be ∽85% effective against severe monkeypox disease (66). As with smallpox, individuals who had been vaccinated as children were still infected with MPXV during the 2003 US outbreak, but they presented with mild or no disease (87, 119). The smallpox vaccine is likely efficacious to various degrees against other orthopoxviruses but not against poxviruses from other genera.

Due to the threat of bioterrorism, the United States developed a new smallpox vaccine, ACAM2000, as a replacement for the limited and aging stocks of Dryvax. ACAM200 was FDA approved in 2007 and is the smallpox vaccine stockpiled in the United States (161). This vaccine was developed from Dryvax and is administered by bifurcated needle. In three phase I clinical trials, ACAM2000 produced major cutaneous reactions, evoked neutralizing antibody and cell-mediated responses, and had a reactogenicity profile similar to that of Dryvax (162). Similarly, phase II randomized, double-blinded, controlled trials found ACAM2000 to be equivalent to Dryvax in terms of cutaneous response rate, antibody responses, and safety (163). In phase III clinical trials, five cases of myo/pericarditis were observed in 873 subjects getting ACAM2000 for the first time, although this was not unexpected, because several cases of myo/pericarditis were identified after smallpox vaccination in three Dryvax and one ACAM2000 phase II clinical trial held in 2003 (164).

A significant proportion of the American population has contraindications for prophylactic vaccination with ACAM2000, because they are immunosuppressed, have significant underlying heart disease, or have a history or presence of atopic dermatitis. Consequently, a more attenuated live vaccinia virus vaccine, called modified Ankara virus (MVA) vaccine, is under evaluation. Since 1968 MVA has been safely used in more than 100,000 humans without

documentation of the adverse reactions associated with other VACV vaccines. Following prophylactic intramuscular or subcutaneous MVA immunization, mice and cynomolgus monkeys are protected from intranasal VACV (165, 166) and intravenous MPXV (165, 167) challenges, respectively. IMVAMUNE, an MVA vaccine (strain BN; Bavarian Nordic GmbH), has been tested for safety and immunogenicity in adult volunteers (168, 169), and may become a next generation smallpox vaccine approved by the FDA. Because the MVA virus does not produce infectious progeny virus in human cells, one or two vaccine doses containing approximately 100 times more MVA virus than ACAM2000 may be required to induce equivalent immune responses and protection.

Dryvax, ACAM2000, and MVA induce cell-mediated and antibody responses against many poxviral antigens (170). Animal models suggest that antibodies targeting a limited number of antigens on the surface of intracellular mature virus and extracellular enveloped virus may be sufficient for protection from disease on challenge because they protect against dissemination of virions through the body (171–173).

Currently, vaccination is recommended for laboratory workers who directly handle cultures or animals infected with VACV or recombinant VACV or other orthopoxviruses that infect humans (174; http://www.bt.cdc.gov/). Stocks of VACV vaccine for immunization of laboratory personnel are available from the Centers for Disease Control and Prevention in Atlanta, GA. To prevent accidental infection of unvaccinated contacts, especially those who are immunosuppressed or suffering from atopic dermatitis, the scarified area is "sealed" with an occlusive bandage.

Management of Outbreaks

The approach to control a human orthopoxvirus that infects via respiratory droplets would depend to a large extent on the size of the initial focus and the reproduction rate of the agent (i.e., the average number of secondary cases generated by a typical primary case in a susceptible population). Although the transmission chains are becoming longer, MPXV still cannot maintain itself in the human population without constant reintroduction from zoonotic hosts. A natural outbreak, or bioterrorist release, of MPXV could be adequately contained with the traditional tracing and vaccination of contacts ("ring vaccination") and the isolation of symptomatic cases. The response to a bioterrorist release of VARV could also be a ring vaccination or the vaccination of large segments of the population and the restriction of civil liberties, depending on the initial burden of cases and the reproduction rate.

TREATMENT

Supportive and Local Care

For nonsystemic zoonotic poxvirus infections, supportive care is provided on an outpatient basis. Invasive treatment procedures are applicable only to MCV. For MCV, the umbilicated core can be removed surgically by curettage, or the lesions may be treated by cryotherapy. This often leads to their resolution and halts the spread to other areas of the skin. These approaches are not appropriate for large numbers of lesions. Cantharidin and topical imiquimod are also commonly utilized agents, although efficacy has not been demonstrated in randomized double-blinded clinical trials (96). Lesions in children can continue to spread to new areas but will ultimately disappear as the children grow older. In immunosuppressed patients, lesions can become more numerous and persist for long periods of time.

A variety of investigational therapies, usually delivered topically, have been used for VACV keratitis, which is estimated to complicate about 30% of ocular vaccinia cases. In a rabbit model of VACV keratitis, intravenous VIG did not affect the course of the disease, whereas topical trifluridine had an antiviral and beneficial clinical effect (175). Topical corticosteroids were associated with rebound in viral replication in this model and should probably be avoided. Thus off-label use of 1% trifluridine is recommended to treat VACV keratitis.

Systemic disease, such as monkeypox, requires hospitalization in severe cases. In the 2003 US outbreak, 9 of 34 patients with laboratory-confirmed monkeypox and no preexisting medical conditions were hospitalized as inpatients. Two were managed in the intensive care unit with respiratory complications (88).

Antiviral Treatment

During the smallpox eradication program, a number of compounds were shown to have efficacy against orthopoxvirus infections in cell culture, but few were actually tested in field conditions. Thiosemicarbazone and metisazone were administered prophylactically in a series of trials in India and showed some protective effect, although their use was often associated with severe nausea and vomiting (2). Cytosine arabinoside and adenine arabinoside were also used to treat variola major and variola minor, but the drugs failed to affect the case mortality rate or the clinical progression of disease. Rifampin showed antiviral activity against VACV in a mouse model but was never tested clinically against VARV.

Cidofovir (CDV), a DNA polymerase inhibitor currently used to treat cytomegalovirus retinitis in AIDS patients, has been approved to treat complications from VACV vaccination under an investigational new-drug protocol; however, it must be given intravenously and has nephrotoxicity (http://www.bt.cdc.gov) (see Chapter 12). This drug has been used off-license (176) to treat ORFV (99) and MCV (177) in immunocompromised patients.

Two orally administered antivirals, although not yet licensed, are part of the United States Strategic National Stockpile. Chimerix and SIGA, Inc., have received investigational new-drug status for the compounds brincidofovir (formerly CMX001) and tecovirimat (formerly ST-246), respectively. Brincidofovir is a hexadecyloxypropyl (HDP)-CDV salt synthesized by covalently coupling CDV to an alkoxyalkanol to form a prodrug of CDV (178). Brincidofovir (200 mg once weekly or 100 mg twice a week) is 80% bioavailable, distributed to tissues without significant concentration in the kidneys, and has lacked nephrotoxicity in clinical studies to date (see Chapter 12). Most importantly, CDV and brincidofovir have broad-spectrum activity against viruses that encode DNA polymerases, including all poxviruses known to infect humans, adenoviruses, and many herpesviruses (179), and brincidofovir has been tested in clinical trials against non-poxvirus viruses (180–182).

Tecovirimat (400 mg per day) is active against multiple species of orthopoxviruses, including two strains of VARV (39, 183). Resistance-mapping studies indicate that it targets the VACV F13L gene, which is conserved in orthopoxviruses. The F13L gene encodes a major envelope protein, p37, which is required for production of extracellular, but not intracellular, virus. Thus, tecovirimat does not affect the actual production of infectious virus; it affects only its

efficient egress from cells. Because brincidofovir and tecovirimat target different stages in the virus replication cycle, they show potent synergy in standard mouse orthopoxvirus infection models (184).

Both of these antivirals will likely treat all human orthopoxvirus infections, and brincidofovir might be effective against the remaining poxviruses, because the DNA polymerase is highly conserved. Consistent with this, CDV diphosphate, which is the active metabolite for both CDV and HDP-CDV, was shown to have activity against MCV DNA polymerase (185). The effectiveness of the egress inhibitor tecovirimat against poxviruses of other genera will depend on the importance of the cell-to-cell spread of virus in the disease process and the presence of the viral target.

While these new drugs have been used to treat a number of serious complications from vaccinia virus vaccine, the drugs and VIG-IV were most intensively studied in a patient who developed progressive vaccinia after developing, and being treated for, acute myelogenous leukemia (186, 187). While acutely ill, this patient required 30 times more VIG-IV than what was estimated to be needed to treat a person with a complication from smallpox vaccination. This may indicate that VIG may not be very effective at treating progressive vaccinia. This patient also required 3 times the dose of tecovirimat than had been used in healthy volunteers in whom pharmacokinetics had been defined. This may indicate that the drug doses may need to be monitored and adjusted in acutely ill patients, especially if ever used to treat someone with smallpox.

Emergence of Resistance and Its Implications

Data available on the emergence of resistance to antiviral treatment mainly come from cell culture and animal models. Resistance to CDV in VACV can be generated after successive tissue culture passage in the presence of a drug or through the introduction of mutations through recombinant DNA technology (188–190). The mutations necessary to make the poxvirus polymerase resistant to CDV in tissue culture appear to reduce the virulence of the virus in tested animal models (189, 191). Resistance to tecovirimat occurs due to mutations in F13L gene (183, 192). While there appears to be a high barrier to the generation of virus resistant to brincidofovir's parental drug, CDV, this is not the case with tecovirimat. Cowpox virus mutants that are resistant to tecovirimat exist in laboratory stocks of virus without the need for drug selection. Mutant virus can be isolated by plaquing the virus in the presence of 10 μM (200 times the EC_{50}) of tecovirimat (183), although other selection conditions took longer to detect resistant virus (192). These trends were seen in the patient being treated for progressive vaccinia (187). In this critically ill patient, resistance to tecovirimat was seen in virus sequentially grown from the vaccination site. Thus, caution is needed when using tecovirimat as monotherapy, especially in a severely ill patient where drug levels may be suboptimal (187).

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Herpes Simplex Viruses

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20

"A system. . . is exactly the opposite of a machine in which the structure of the product depends crucially on strictly predefined operations of the parts. In the system the structure of the whole determines the operation of the parts; in the machine the operation of the parts determines the outcome."

Paul Alfred Weiss, in A. Koestler, V. R. Smithies eds., *Beyond Reductionism*,1968.

London Hutchinson

Herpes simplex virus (HSV) infections of humans have been documented since the advent of writing. Genital herpes was described in Sumerian literature (1). Greek scholars, particularly Hippocrates, used the word "herpes," meaning to creep or crawl, to describe the spreading nature of skin lesions (2). Herodotus associated mouth ulcers and lip vesicles with fever and called it "herpes febrilis." Many of these original observations likely emanated from Galen's deduction that the appearance of lesions was an attempt by the body to rid itself of evil humors, leading to the description "herpes excretins."

As noted by Wildy (2), Shakespeare wrote of recurrent HSV labial lesions. In Romeo and Juliet, Queen Mab, the midwife of the fairies, stated: "O'er ladies lips, who straight on kisses dream, which oft the angry Mab with blisters plagues, because their breaths with sweetmeats tainted are." In the 18th century, Jean Astruc, physician to King Louis XIV, associated herpetic lesions with genital infection. By the early 19th century, the vesicular nature of lesions associated with herpetic infections was well characterized. However, it was not until 1893 that Jean Baptiste Émile Vidal specifically recognized person-to-person transmission of HSV infections (2).

By the beginning of the 20th century, histopathologic studies described multinucleated giant cells associated with infection, and the infectivity of HSV was established by transmission of virus from humans to the cornea of the rabbit, resulting in keratitis. Reports through the 1950s focused on the biologic manifestations of HSV infection and the natural history of human disease. The host range of HSV was expanded to include a variety of laboratory animals, chick embryos, and cell culture systems. Expanded animal studies demonstrated that transmission of human virus could occur not only to the rabbit, as noted above, but also could lead to infections of the skin or the central nervous system (3, 4).

Host immune responses to HSV were defined by the presence of neutralizing antibodies to HSV in the serum of newly and previously infected children and adults (5). Subsequently, some seropositive individuals developed recurrent labial lesions, albeit less severe than those associated with the initial episode. These observations, contrary to all known interactions of humans with infectious agents, led Robert Doerr, a leading virologist of the past century, to postulate that "All of these observations converge to indicate that the agent of herpes is no infectious agent which is conserved in the site of infection, but that it originates in the human organism, that is, endogenously" (6).

HSV has the distinctive property of establishing a silent or "latent" infection and reactivating in the presence of humoral and cell-mediated immune responses. In the course of these studies, the spectrum of disease was expanded to include primary and recurrent infections of mucous membranes (e.g., gingivostomatitis, herpes labialis, genital HSV infections, etc.), keratoconjunctivitis, neonatal HSV infection, visceral HSV infections of the immunocompromised host, HSV encephalitis, Kaposi's varicella-like eruption, and an association with erythema multiforme.

Among many important laboratory advances of the past century was the detection of antigenic differences between HSV types (7). Historically, HSV-1 was more frequently associated with nongenital infection (infection above the waist), whereas HSV-2 was associated with genital disease (infection below the waist). This knowledge provided a foundation for many of the studies performed during the latter part of the 20th century. Over the past two decades, both HSV-1 and HSV-2 can be found in the mouth and in the genital tract, with HSV-1 becoming a more common resident of the genital tract.

The replication cycle of HSV in humans consists of three phases: the replication phase, in which HSV actively replicates and spreads from cell to cell; the latency phase, in which with few exceptions viral genes are silent, or at best, randomly transcribed at a very low frequency; and the reactivation phase in which the virus recurs from dormancy, replicates,

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and is transported anterogradely to a site at or near the initial infection to reinitiate the replication phase (8).

The characterization of an infectious agent as a pathogen must take into account that the mission of all viruses is to spread and multiply. The task facing HSV is formidable: Spread from person to person is by voluntary contact between infected mucous membranes of one individual with those of an uninfected individual. Deceased individuals and people without obviously grotesque lesions do not transmit, as reviewed (9). Notwithstanding these limitations, HSV is one of the most successful human pathogens. In most societies, its penetration is 100% of the population, and it persists in a human for life. Numerous studies carried out in recent years suggest that it accomplishes its mission by tightly regulating its growth and resultant morbidity.

VIROLOGY

Classification

Herpes simplex viruses belong to the family Herpesviridae (10). The >100 known members of this family share the structure of their virions, general features of their reproductive cycle, and the capacity to remain latent. They differ, however, in many respects, and have been classified into three subfamilies (alpha, beta, gamma) with respect to the details of their replication, the cells in which they remain latent, gene content, and gene organization. HSV types 1 and 2 belong to the subfamily Alphaherpesviridae (11).

Virion Structure

The HSV virion consists of four concentric components. From the center out, these are: (1) an electron-dense core containing viral DNA and polyamines, (2) an icosadeltahedral capsid consisting of capsomeres (12), (3) an amorphous layer of proteins, designated tegument, surrounding the capsid, and (4) an envelope containing lipids and studded with at least 10 viral glycoproteins and several nonglycosylated membrane proteins.

Herpes simplex viruses have genomes that consist of a single double-stranded DNA molecule (12, 13), which is in excess of 152 kbp (the exact size depends on the number of terminal repeats). The G+C content of HSV-1 DNA is 68% and 69% for HSV-2 DNA. The HSV-1 and HSV-2 genomes encode at least 84 different polypeptides (12, 14). The DNAs consist of two covalently linked components, Large and Small (L and S), each consisting of stretches of unique sequences designated U_L and U_S that are flanked by the inverted repeats ab and ca, respectively. The two components invert relative to each other to yield four populations of molecules differing solely in the relative orientation of U_S and U_L (15). Although they differ with respect to nucleotide sequence and restriction endonuclease cleavage sites, and in apparent sizes of many viral proteins they encode, the HSV-1 and HSV-2 genomes are collinear and readily form intertypic recombinants in the laboratory (15).

Proteins

HSV encodes at least 100 transcripts, of which 84 are translated into unique proteins. Of this number, 46 are dispensable for viral replication in at least some cultivated cells. The function of many of these "dispensable" genes is to render viral replication and spread more efficiently or to block host responses to infection (12, 16). Importantly, these genes are not truly dispensable, as viruses lacking these genes have not been isolated from humans. Viruses from which these genes have been deleted by genetic engineering frequently exhibit a reduced capacity to multiply and spread in experimental animals (12, 16). Indeed, the observation that many HSV genes are dispensable and can be replaced by non-HSV genes sustains the expectation that HSV can be designed to perform different functions, such as to serve as vectors for foreign gene expression, live attenuated vaccines, or for selective destruction of cancer cells.

Because viral proteins studied to date have been found to perform multiple functions, it is likely that HSV encodes several hundred functions expressed by adjacent or overlapping amino acid blocks. Each function is performed at a designated time during replication and at an intracellular location that may be determined by posttranslational modifications and by protein partners that interact selectively with these posttranslationally modified viral proteins. Modifications include phosphorylation by cellular (e.g., cdc2) or viral (U_L13, U_S3) protein kinases, nucleotidylation by casein kinase 2, poly(ADP)-ribosylation, among others. U_L13, a key viral protein kinase, is homologous to the U_L97 protein kinase of human cytomegalovirus (CMV) and a key component of antiviral chemotherapy (10).

Replication

In recent years, it has become apparent that the replication of HSV is defined in part by both intrinsic mechanisms encoded by the virus and host innate immune responses. It is convenient and to some extent necessary to describe these processes in parallel (10).

Initiation of Replication

To initiate infection, HSV must attach to cell-surface receptors and fuse its envelope with the plasma membrane or the membrane of the endocytic vesicle (Fig. 1). Viral surface glycoproteins mediate attachment and penetration of the virus into cells. They also elicit a "protective" host immune response to the virus. Of the 12 viral glycoproteins (g) designated gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, gM, and gN, five are involved in the entry process. In brief, gB and gC enable virions to attach to heparin-sulfated proteoglycans that stud the cell surface. In the next step, gD interacts with its receptor and triggers the fusogenic functions of gB and gH/gL. The consequence of the fusion of the envelope with the cellular membranes is the release of the capsid and approximately 20 tegument proteins into the cytoplasm. The capsid is transported to the nuclear pore, where the DNA is released from the capsid into the nucleoplasm. At least some tegument proteins are also transported into the nucleus. Little is known of the function of viral glycoproteins other than gC, gB, gD, and gH/gL. gE and gI form a potent Fc receptor that binds immunoglobulins at the cell surface.

To date, three cellular receptors have been described. These are: (1) HveA, a member of the tumor necrosis factor-α (TNF-α) receptor family; (2) Nectin1, a member of the extended immunoglobulin family; and (3) a modified heparin sulfated proteoglycan. Of the three, Nectin 1 is the major receptor enabling infection of human cells with HSV (10).

Postentry Events

It is convenient to define three distinct phases of postentry events: immediate early, early, and late (Fig. 1). First, a complex of nuclear proteins aggregate at the entering viral DNA and form a complex designated ND10. The organizer of this complex is promyelocytic leukemia (PML) protein. Concurrently, viral DNA is silenced by deacetylated histones and methylation. Among the structures bound to the

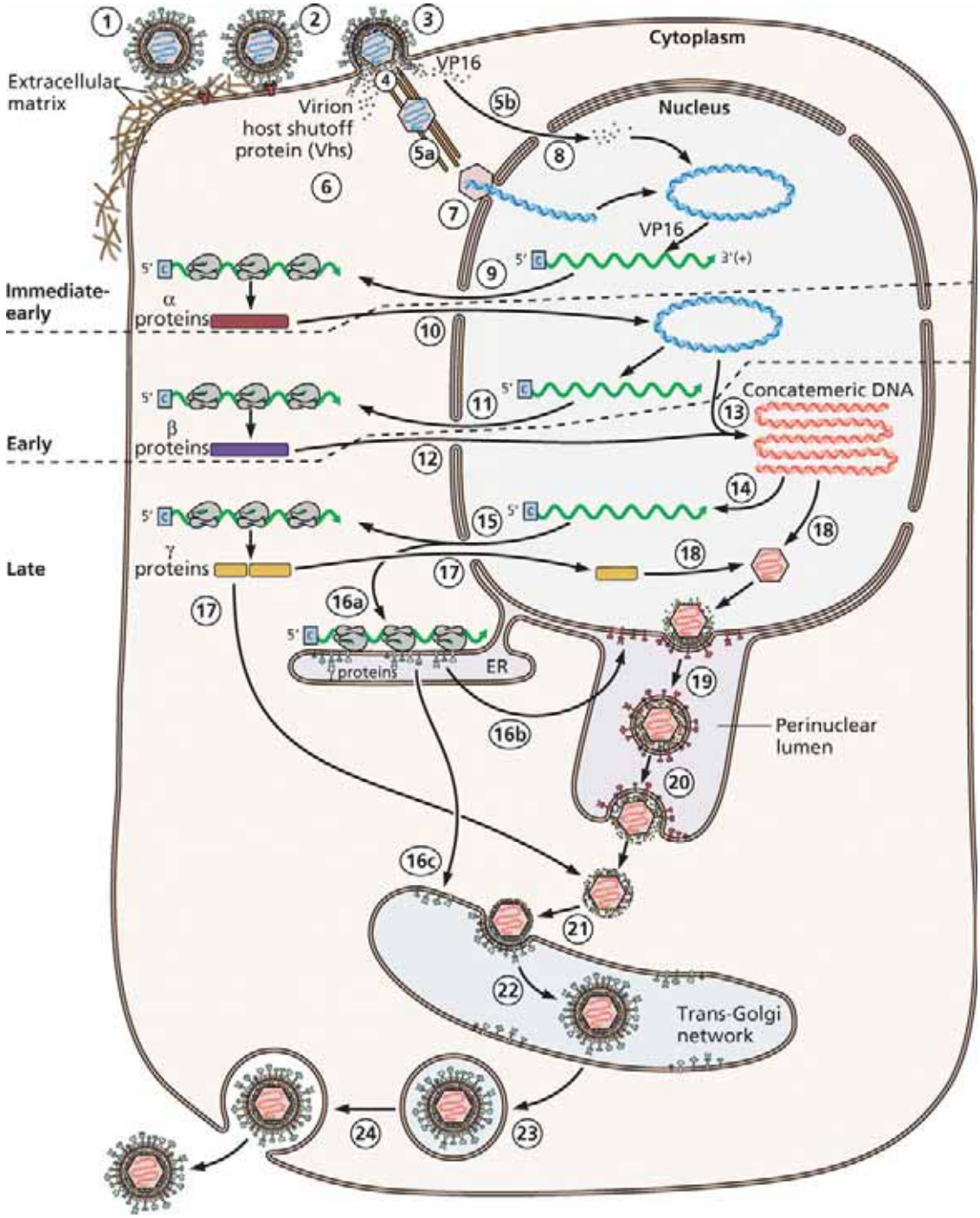

FIGURE 1 Schematic representation of the replication of herpes simplex viruses (HSV) in susceptible cells. 1. The virus initiates infection by the fusion of the viral envelope with the plasma membrane following attachment to the cell surface. 2. Fusion of the membranes releases two proteins from the virion. Virus host shutoff (VHS) shuts off protein synthesis (broken RNA in open polyribosomes). The α gene trans-inducing factor (α-TIF) is transported to the nucleus. 3. The capsid is transported to the nuclear pore where viral DNA is released into the nucleus and immediately circularizes. 4. The transcription of α genes by cellular enzymes is induced by α-TIF. 5. The 5 αmRNAs are transported into the cytoplasm and translated (filled polyribosome); the proteins are transported into the nucleus. 6. A new round of transcription results in the synthesis of ß proteins. 7. At this stage in the infection the chromatin (c) is degraded and displaced toward the nuclear membrane, whereas the nucleoli (round hatched structures) become disaggregated. 8. Viral DNA is replicated by a rolling circle mechanism, which yields head-to-tail concatemers of unit length viral DNA. 9. A new round of transcription/translation yields the γ proteins consisting primarily of structural proteins of the virus. 10. The capsid proteins form empty capsids. 11. Unit-length viral DNA is cleaved from concatemers and packaged into the preformed capsids. 12. Capsids containing viral DNA acquire a new protein. 13. Viral glycoproteins and tegument proteins accumulate and form patches in cellular membranes. The capsids containing DNA and the additional protein attach to the underside of the membrane patches containing viral proteins and are enveloped. 14. The enveloped proteins accumulate in the endoplasmic reticulum and are transported into the extracellular space. Reprinted with permission from (192).

viral DNA is the histone deacetylase 1 (HDAC-1) or HDAC-2 CoREST/ LSD1/REST repressor complex. This complex silences neuronal genes in nonneuronal cells. By extension, nonneuronal cells perceive HSV genes as neuronal. To express its function, viral DNA must be derepressed (17, 18).

Three key proteins are among the tegument components entering the nucleus. These are VP16, the virion host shutoff RNase (VHS-RNase) and pU_L47. To initiate replication, VP16 recruits several transcriptional factors, including octomer binding protein 1 (Oct1), host cell factor 1 (HCF1), and lysine-specific demethylase 1 (LSD1) to the promoters of α [immediate early (IE) genes] (19). The six IE or α proteins [infected cell protein (ICP) 0, 4, 22, 27. 47 and US1.5] are expressed first. Next, ICP0 performs several functions. The functions important at this stage are 2-fold. First, ICP0 binds to CoREST and dislodges HDAC1. In consequence, the HDAC1 or 2/CoREST/LSD1/REST repressor complex falls apart and is translocated to the cytoplasm (20–22). This enables the early or β genes to be transcribed. Second, ICP0 recruits to the transcriptional machinery key cellular proteins (e.g., CLOCK, etc.) essential for efficient transcription of viral genes (23). The α proteins have two key functions. Foremost, they disable, largely by preemptive actions, host responses to intrusion of foreign DNA and proteins into the infected cells. Equally important, they insure efficient expression of viral genes through subversion and recruitment of host proteins (8).

The key function of β gene products is to replicate viral DNA. HSV expresses at least seven proteins known to play a role in viral DNA synthesis, but additional host proteins may also be involved. The proteins involved in viral DNA synthesis are the targets of anti-HSV drugs (10). Once replication of viral DNA begins, the late or γ genes are expressed. Most γ proteins are structural components of the virion.

Assembly of the capsid and insertion of the viral DNA into the preformed capsid takes place in the nucleus. Envelopment takes place at the inner nuclear membrane and in the multivesicular bodies in the cytoplasm. Precisely how capsids reach the multivesicular bodies is the subject of much debate. One theory suggests that virions formed at the inner nuclear membrane become de-enveloped at the outer nuclear membrane or in vesicles formed by the outer membrane. The opposing view is that capsids are transported out of the nucleus through the nuclear pores, which fluctuate in size depending on the size of the transient cargo. Virions are transported to the cell surface in vesicles derived either from the outer nuclear membrane or the multivesicular bodies (10).

HSV Replicative Functions and the Host Innate Immune Responses

In parallel with viral gene expression, a multifaceted interplay takes place between viral gene products and the host, one result of which is degradation of mRNAs encoding host response gene products. Cell sensors detect entry of virus into cells and induce the transcription of several hundred genes encoding stress response mRNAs that characteristically contain one to several AU-rich elements (AUUUUA) in their 3'-untranslated region (3'-UTR) and have a relatively short half-life. Thus, in uninfected cells, the stress response mRNAs are ultimately bound at the AU-rich elements by a protein (e.g., tristetraprolin) and are degraded in p-bodies or sequestered in exosomes. In HSV wild-type virus-infected cells, three series of events take place (24, 25). First, VHS-RNase, a tegument protein, binds to the Cap structure of preformed cellular mRNAs and cleaves them at or near the Cap structure. The Cap-less mRNA is rapidly degraded 5' to 3' by cellular enzymes. Second, VHS-RNase and pU_L47, another tegument protein, bind to the Cap structure of mRNAs made after infection. These mRNAs, of both cellular and viral origin, are not degraded except for the stress response mRNAs. The exception is the stress response mRNAs, in this instance among the host proteins induced after infection is tristetraprolin (26). Third, tristetraprolin forms a complex with VHS-RNAse, binds to the AU-rich elements of the stress response mRNAs, and cleaves the 3'-UTR (27). The cleaved 5' portion, which contains the open reading frame, persists in the cytoplasm for many hours. Notably, the VHS-RNase-tristetraprolin complex does not degrade all stress response mRNAs. Among the mRNAs spared are those that encode tristetraprolin and ATF3.

HSV infection also causes degradation of PML and blockade of interferon (IFN) sensitivity. A key function of ICP0 is that of an ubiquitin ligase. In conjunction with UbcH5A ubiquitin conjugating enzyme, ICP0 mediates the degradation of the PML protein and SP100—two key components of ND10 (28–30). A key consequence of the degradation of PML is the loss of sensitivity to IFN (31). Thus, formation of ND10 bodies associated with viral DNA has the objective of turning off the viral transcriptome (17). HSV takes advantage of the association with ND10 to recruit ICP0 to PML ultimately rendering the infected cell insensitive to IFN. It also serves as a locus for assembly of transcriptional factors that ultimately enable the synthesis and assembly of viral particles (19). ICP0 E3 ligase activity has been reported to be responsible for the degradation of other proteins, notably that of IF116, a nuclear host DNA sensor (32).

The presentation of antigenic peptides is inhibited at the cell surface. ICP47, an α protein, binds Tap1/Tap1 and blocks the transport and presentation of antigenic peptides on the surface of infected cells. In essence, the infected cell is invisible to the cellular adaptive immune system until late in infection (10).

Dephosphorylation of Eukaryotic Initiation Factor 2α by ICP34.5

Beginning with the synthesis of γ proteins, there is an increase in the accumulation of complementary transcripts capable of forming double-stranded (ds) RNA. The presence of dsRNA activates protein kinase R (PKR), which, in turn phosphorylates the translation initiation factor eukaryotic initiation factor 2α (eIF-2α). In turn, eIFα-p shuts off protein synthesis, leading to cell death. To overcome this block, ICP34.5 encoded by the $\gamma_1$34.5 gene recruits cellular protein phosphatase-1α to dephosphorylate eIFα-p and enable the synthesis of late viral proteins (33).

The Latency Phase (10, 18, 34, 35)

The most common sites of latent infections are sensory or dorsal root neurons, although latent infections may also be established in autonomic neurons. The key property of these neurons is that they are shrouded by satellite cells. The affected neurons are anchored at one end in the mucous membranes, namely sites of initial HSV infection, and at the other by communicating neurons of the autonomic or central nervous system. HSV accesses sensory neurons by infection of nerve endings at the site of replication. The capsids containing viral DNA are then transported retrogradely in axons to the nuclear pore. The sequence of events

is known mostly from studies of murine models. In mice infected by the corneal scarification, the virus can be detected in trigeminal ganglia in less than 24 hours. The sequence of events is highly reproducible. HSV replicates in neurons, and infectious virus can be detected for at least 2 weeks. By week 3, infectious virus begins to disappear. What remains are neurons harboring latent virus. Infectious virus readily appears (reactivates) on excision of the ganglia and cultivation of the neurons *ex vivo*.

Several aspects of HSV infection that lead to latency are different from those that lead to replication. Foremost, latent infections cannot be established in cell culture or in tissues at the peripheral sites of viral replication. One hypothesis that could explain the restricted repertoire of cells capable of maintaining HSV in the latent state is that HCF1 in neurons is in the cytoplasm and that both VP16 and HCF are either not transported to the nucleus of the infected neurons or the transport is retarded (36, 37). As is the case of cells infected at the peripheral sites, on entry into the nucleus, viral DNA is coated by repressive proteins and essentially silenced (17). The only domain of HSV expressed at high levels consists of a 9-kb DNA stretch expressing a noncoding RNA that is processed by splicing into a stable intron designated the latency-associated transcript or LAT (38, 39).

The domain encoding the LAT precursor is enclosed by DNA sequences that insulate it from the remainder of the viral genome (40). The remainder of the DNA is transcribed randomly at very low levels. In essence, during the latency period, viral DNA is in the form of heterochromatin whose state is in a dynamic equilibrium defined by numerous factors. Stimulation of the neuron by injury [e.g., ultraviolet (UV) light], cytokines [e.g., interleukin-6 (IL6)] and other factors induce a stress response that can lead to apoptosis. Maintaining the viability of the neuron and at the same time silencing viral DNA are LAT, nerve growth factor (NGF), histone deacetylases, signal transducer and activator of transcription 3 (STAT3), the phosphatidylinositide 3-kinase/AKT (PI3K-AKT) pathway, and stress response factors (41, 42). LAT plays an important role in the viability of the neurons harboring latent virus and at the same time maintains the virus in latent state. Reactivation results from stimulations that lead to apoptosis and which cannot be blocked (43).

The Reactivation Phase

The most common causes of reactivation of HSV are exposure to UV light, fever, emotional or physical stress, and menstruation, as discussed below. Experimentally, reactivation may also be induced by neuronal injury, deprivation of NGF or proapoptotic drugs (23, 42). A key question is the mechanism by which viral DNA is derepressed to enable its transcription in the absence of VP16 and ICP0. Recent studies show that nerve injury induces a proapoptotic response, but also concurrently activates the STAT3 and PI3K-AKT pathway to induce a stress response that suppresses apoptosis and blocks reactivation (42). A strong proapoptotic stimulus triggers total derepression of viral DNA. In essence, all viral genes, including regulatory proteins, viral DNA, and structural proteins, are synthesized simultaneously. The consequences are that the virus yields in reactivating neurons are quite small—enough to be transported anterogradely to peripheral tissues but not enough to pose a major threat to the host (23).

The frequency of reactivation, namely a higher number, has been linked to a specific chromosomal locus for labial herpes. This locus is on chromosome 21 and is known as the Cold Sore Susceptibility Gene (CSSG-1) (44). There are, however, no known links between the chromosomal locus and the events taking place in neurons harboring latent virus.

Control of Replication, Latency, and Reactivation

The ability of HSV to replicate and cause extensive lesions with primary infection, to establish latent infections, and then reactivate and again cause lesions in the presence of adaptive immunity, along with the high rate of penetration in the human population, are at first glance the properties of a successful pathogen. A central question then is how does the virus control its replication?

Recent studies suggest that HSV evolved novel functions that limit growth and cell-to-cell spread of virus. The findings that support this hypothesis are:

(1) Activation of PKR leads to induction of nuclear factor-kappaB (NF-kappaB) and ultimately to activation of IFN-stimulated genes. In infected cells, PKR is blocked primarily by dephosphorylation of eIF-2α-p (33). Direct suppression of PKR does not take place, notably by U_S11, until relatively late in infection (45). At the same time deletion of NF-kappaB p60 or p55 or both p60 and p55 components results in significantly lower yields following infection with wild-type virus (46).

(2) Activation of the stimulator of IFN genes (STING) by nuclear or cytoplasmic DNA sensors leads to synthesis of IFN and of IFN-stimulated genes. Curiously, STING is effectively stabilized in wild-type virus-infected cells and is exported in exosomes from infected to uninfected cells (47).

(3) To date, 30 virally encoded microRNAs (miRNAs) have been detected in productively infected cells, in ganglia harboring latent virus, and in ganglia in which HSV was induced to reactivate from its latent state. With few exceptions, miRNAs made during productive infection also accumulate in ganglia harboring latent virus, but not in ganglia in which HSV is in the process of reactivation. In contrast, the miRNAs accumulating in ganglia in which HSV is reactivating do not accumulate in productively infected cells and are absent from ganglia harboring latent virus. One hypothesis that could explain this paradox is that miRNAs suppress viral replication in ganglia in which the virus replicates and suppress some aspect of innate immunity, perhaps proapoptotic functions, in productively infected cells and ganglia harboring latent virus. Thus, two of the miRNAs accumulating in trigeminal ganglia harboring reactivating virus accumulate very late in the course of productive infection, i.e., at the time when the bulk of infectious virus had already been made. Studies in progress indicate that they have a significatnt inhibitory effect on viral gene expression when expressed before infection. Consistent with a role in controlling viral replication and spread, some of the miRNAs are exported in exosomes from infected to uninfected cells (9, 48).

(4) Last, the HDAC-1 or HDAC-2/CoREST/LSD1/REST repressor complex acts by binding of REST to RE1 response elements in viral DNA. Insertion into viral DNA of a dominant negative REST that binds RE1, but cannot bind the other components of the repressor complex, enabled the recombinant virus to replicate to higher levels and be more virulent in mice than the wild-type parent virus (49).

The examples cited above suggest that HSV has evolved mechanisms to replicate to higher levels and to spread more efficiently from infected to uninfected cells by eliminating its dependence on active NF-kappaB, eliminating RE1 sites and blocking export of STING and miRNAs from infected to

uninfected cells. A necessary conclusion of these observations is that HSV has evolved mechanisms to control its replication and spread to sustain a high level of person-to-person transmission.

Pathogenic Correlates of Viral Replication

HSV-1 and HSV-2 exhibit two unique biologic properties that influence human disease. Both viruses have the capacity to invade and replicate in the central nervous system (CNS) and, as noted above, the ability to establish a latent infection (13).

The term *neurovirulence* encompasses both the ability of HSV to invade the CNS from peripheral sites and to replicate in neuronal cells. When paired isolates (brain and lip) from patients with HSV encephalitis are evaluated by plaque-forming unit/median lethal dose (PFU/LD_{50}) ratios following direct intracerebral inoculation in mice, encephalitis isolates have lower PFU/LD_{50} ratios than isolates from lip lesions, ergo are more neurovirulent. Neurovirulence appears to be the function of numerous genes (12). In fact, deletion of virtually any of the genes dispensable for viral replication in cell culture reduces the capacity of the virus to invade and replicate in CNS. Mutations affecting neuroinvasiveness have also been mapped in genes encoding glycoproteins. Access to neuronal cells from usual portals of entry into the body requires postsynaptic transmission of virus, and therefore a particularly vigorous capacity to multiply and to direct the virions to appropriate membranes. In addition, because neuronal cells are terminally differentiated and do not make cellular DNA, they lack the precursors for viral DNA synthesis that are also encoded by the viral genes dispensable for growth in cell culture. Of particular interest, however, is the role of the $\gamma_1$34.5 gene in neurovirulence (50–53). Although $\gamma_1$34.5 deletion mutants multiply well in a variety of cells in culture, they are among the most avirulent mutants identified to date when inoculated directly into the brain of susceptible animals.

Latency has been recognized biologically since the beginning of the century (54–57). Latent virus can be retrieved from the trigeminal, sacral, and vagal ganglia of humans either unilaterally or bilaterally (55). The recovery of virus by *in vitro* cultivation of trigeminal ganglia helps explain the observation of vesicles that recur at the same site in humans, usually the vermilion border of the lip. Patients treated for trigeminal neuralgia by sectioning a branch of that nerve develop herpetic lesions along the innervated areas of the sectioned branch (58–61). Reactivation of latent virus appears dependent upon an intact anterior nerve route and peripheral nerve pathways. Recurrences occur in the presence of both cell-mediated and humoral immunity. Recurrences are spontaneous, but there is an association with physical or emotional stress, fever, exposure to UV light, tissue damage, and immune suppression. Recurrent herpes labialis is three times more frequent in febrile patients than in nonfebrile controls (54, 56, 62, 63).

EPIDEMIOLOGY

Orolabial HSV Infections

Prevalence

Although HSV-1 and HSV-2 are usually transmitted by different routes and may involve different body sites, there is a great deal of overlap between their epidemiology and clinical manifestations. These viruses are distributed worldwide and occur in both developed and underdeveloped countries, including remote Brazilian tribes (64). Animal vectors for human HSV infections have not been described; therefore, humans remain the sole reservoir for transmission to other humans. Virus is transmitted from infected to susceptible individuals during close personal contact. There is no seasonal variation in the incidence of infection. Because infection is rarely fatal and HSV becomes latent, over one-third of the world's population has recurrent HSV infections, and, therefore, the capability of transmitting HSV during episodes of productive infection.

Geographic location, socioeconomic status, and age influence the prevalence of HSV-1 infection (5, 65). In developing countries and societies, infection, indicated by seroconversion, occurs early in life. Among Brazilian Indians and children in New Orleans, HSV antibodies develop in over 95% of children by the age of 15 (66). In lower socioeconomic populations, approximately one-third of children seroconvert by 5 years of age; this frequency increases to 70–80% by early adolescence. Middle-class individuals in industrialized societies acquire antibodies later in life, such that seroconversion over the first 5 years occurs in 20% of children, followed by no significant increase until the second and third decades of life, during which the prevalence of antibodies increases to 40% and 60%, respectively (67). Seroconversion of susceptible university students occurs at an annual frequency of approximately 5–10% (67).

In the United States, the seroprevalence of HSV-1 and HSV-2 infections varies by age, sex, and race (Fig. 2) (68, 69). Through childhood and adolescence, African Americans have approximately a 2-fold higher prevalence of antibodies to HSV-1 than Caucasians, and females a slightly higher prevalence of HSV-1 antibodies than males. By the age of 40, both African Americans and Caucasians have a similar prevalence of antibodies. A recent global assessment indicated that the point prevalence of HSV-1 infection by age 49 years is 67%, a finding consistent with earlier studies (70). The high prevalence of antibodies appearing later in life may be the consequence of a cohort effect, namely these individuals had a higher rate of acquisition of HSV-1 earlier in life.

Seroprevalence worldwide shows geographic variation. An HSV-1 antibody prevalence less than 70% is found in populations residing in Pittsburgh, Pennsylvania; Birmingham, Alabama; Atlanta, Georgia; Japan; Lyon, France; Sweden and Caucasian Americans [U.S. National Health and Nutrition Examination Survey (NHANES) participants], whereas it exceeds 95% in Spain, Italy, Rwanda, Zaire, Senegal, China, Taiwan, Haiti, Jamaica, and Costa Rica for adults between the ages of 20 and 40.

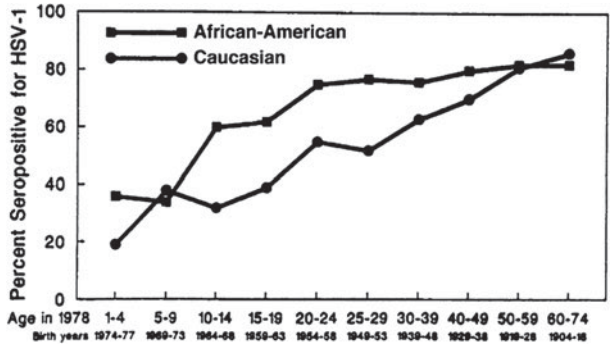

FIGURE 2 Seroprevalence of herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) by age.

Virus can be isolated from the saliva of approximately 20% of asymptomatic children between 7 months to 2 years of age (146). Virus shedding in children less than 6 months of age is uncommon. In older children, 3–14 years of age, presumed asymptomatic shedding has been documented in 18%. Virus recovery decreases with advancing age; over 15 years of age, the frequency of excretion is 2.7%, similar to contemporary cross-sectional surveys, which range from 2% to 5% (135,147–149).

Virologic and Clinical Recurrences

The largest reservoir of HSV infections in the community is recurrent herpes labialis. A positive history of recurrent herpes labialis was noted in 38% of 1800 graduate students in Philadelphia (71). New lesions occurred at a frequency of one per month in 5% of the students and at intervals of 2–11 months in 34% of the students. Recurrences of one per year or less often were found in 61%. Over the past several years, HSV-2 infections of the oropharynx have been recognized both in immune competent and immunocompromised hosts (72).

Outbreaks of HSV-1

Clustered human outbreaks of HSV infections have been reported (65), but there is no indication from either clinical or molecular epidemiologic studies that HSV causes epidemics in the general population. Most of the studies involve families in which several individuals suffer from HSV infection at approximately the same time, perhaps through exposure to recurrent labial lesions of one family member. Outbreaks in healthcare settings have occurred, but no common source can be determined. An outbreak of eczema herpeticum was reported in a large group of hospitalized patients within an 8-day period; in this case, the lack of attention to infection control procedures (e.g., hand-washing) was incriminated as being responsible for virus transmission. Outbreaks of herpetic stomatitis have been reported in dental offices and within orphanages (73), where attack rates for apparent infection were approximately 75% of susceptible children. Increased transmission of HSV in day care centers has not yet been reported (74).

Genital HSV Infections

Prevalence and Incidence

Because genital HSV infections are usually acquired through sexual contact, antibodies to HSV-2, at least, are rarely found before the age of onset of sexual activity (75). Although most recurrent genital HSV infections are caused by HSV-2, an ever-increasing proportion of primary infection is attributable to HSV-1 (76), now accounting for nearly 50% of new cases (77). The differentiation in virus type is significant, because genital HSV-1 infections are usually both less severe clinically and significantly less prone to recur (76, 78). The number of new cases of genital HSV infections has been conservatively estimated to be approximately 500,000 individuals annually. Predicated upon newer serologic methods for detection of prior HSV-2 infection, a range of 40–60 million Americans are infected with HSV-2 (79).

Seroprevalence of HSV-2 increases from 5.6% between 12 and 19 years of age to 24.3% by the age of 60. For the aforementioned age groups, 4.5% and 8.7% of Caucasians, respectively, and 18.2% and 76.8% of African Americans, respectively, have HSV-2 antibodies. Factors found to influence acquisition of HSV-2 include sex (women greater than men), race (African Americans more than Caucasians), marital status (divorced more than single or married), and place of residence (city greater than suburb) (80). In the United States, a 20% increase in seroprevalence, indicative of infection, occurred between NAHNES II (1976–1980) and NAHNES III (1988) (Fig. 3). However, the most recent study (NAHNES IV) indicates slightly lower HSV-2 seroprevalence rates (81). Seroprevalence studies indicated the highest prevalence of antibodies to HSV-2 in the United States is in female prostitutes (75%), and is virtually identical to that of prostitutes in Tokyo (69,82–84). Seropositivity amongst female prostitutes in Dakar, Senegal, was even higher—95.7% in 1985. Homosexual men have seroprevalence rates to HSV-2 varying from a high of 83.1% in San Francisco (1985–1986) to low of 21.6% in Seville, Spain (1985–1986), Tokyo, Japan 24.2% (1988), and Amsterdam, The Netherlands of 50.0% (1986). Although these seroprevalence rates account for HSV-2, they cannot discern the contribution of HSV-1 to genital infection.

Prospective studies of the incidence of HSV-2 infection indicate that among low-risk individuals, namely college students, the rate of acquisition was approximately 2% per year over 4 years as compared to 4% per year for homosexual men in San Francisco (69). The incidence of HSV-2 infection during pregnancy is about 2.5% per gestation, but may be as low as 0.58%. Acquisition of HSV-2 infection between monogamous sexual partners with discordant infection status is 10 to 15% yearly (97).

As noted, the number of different sexual partners, irrespective of sexual preference, correlates directly with acquisition of HSV-2 (Fig. 4) (69, 85). For heterosexual women living in the United States with one partner, the probability of acquisition of HSV-2 is less than 10%, but increases to 40%, 62%, and greater than 80% as the number of lifetime sexual partners increases to 2–10, 11–50, or greater than 50, respectively. For heterosexual men, similar data are 0% for one lifetime sexual partner, and 20%, 35%, and 70% for each of the three risk groups, respectively. In contrast, for homosexual men, seroprevalence increases from greater than 60–90% for those with 11–50 and greater than 50 partners. Thus, multiple sexual partners, irrespective of sexual preference, correlates directly with acquisition of HSV-2 infection (69, 85).

Virologic and Clinical Recurrences

As with HSV-1 infections of the mouth, HSV-2 is excreted more frequently in the absence of symptoms at the time of primary, initial, or recurrent infection (86–88), providing a silent reservoir for transmission. The frequency of clinical

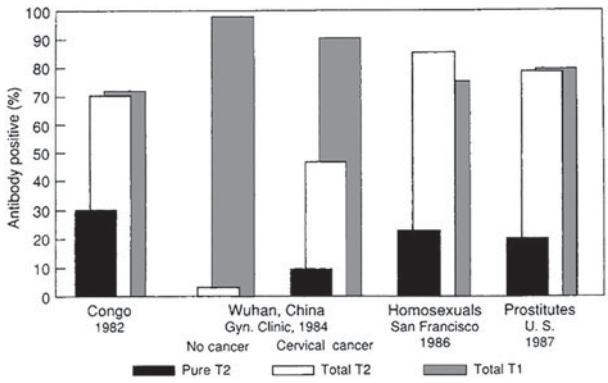

FIGURE 3 Seroprevalence of herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) by selected country.

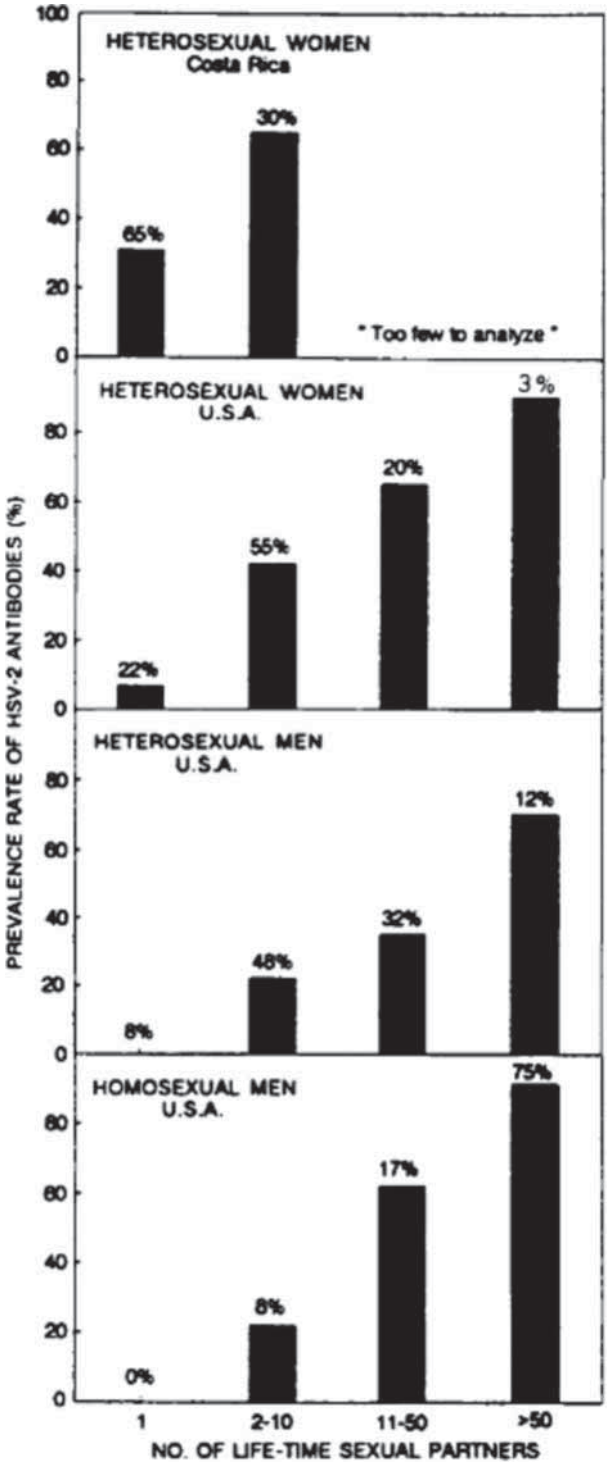

FIGURE 4 Seroprevalence of herpes simplex virus type 2 (HSV-2) as a function of the number of sexual partners.

recurrences varies somewhat between males and females, with rates of 2.7 and 1.9 per 100 days, respectively (76), figures much lower than recurrences detected by laboratory tests, as noted in the following paragraphs. Recurrences are most frequent in the year immediately following acquisition and tend to decrease somewhat over subsequent years. Several studies have implicated a frequency of clinical recurrences as high as 60%. Genital infections by HSV-1 recur less frequently than those caused by HSV-2 (78, 89). Following the first episode of genital herpes, asymptomatic shedding was detected in approximately 12%, 18%, and 23% of women with primary HSV-1, primary HSV-2, and nonprimary HSV-2 infection, respectively (90). For women with established genital HSV-2 infection, asymptomatic shedding was detected on 1% of all days cultures were obtained (88, 91). More contemporary studies indicate that only 20–25% of women who are HSV-2 seropositive have symptoms of genital herpes (92, 93). With the application of the polymerase chain reaction (PCR) to the serial evaluation of genital swabs from women with genital infection, the frequency of shedding of HSV DNA is even higher, suggesting that HSV is a chronic infection rather than an intermittent one (94). Indeed, HSV DNA can be detected in up to 20% of all days for women with HSV-2 infection. Interestingly, shedding can occur for a mere few hours on any given day and multiple sites, as reviewed (95, 96). Similar shedding data will be found for HSV-1 oropharyngeal infection (72).

HIV Coinfection

HSV-2 infection, by the nature of being an ulcerative disease, is associated with acquisition of human immunodeficiency virus type 1 (HIV-1). Most case–control studies performed in the United States and Central Africa indicate increased relative risk of HIV acquisition by 1.5- to over 2-fold (69,98–100). Furthermore, concomitant infection is associated with higher HIV RNA plasma levels that can be decreased with HSV therapy (101, 102). The association between acquisition of HIV and HSV-2 was also documented in heterosexual populations (99).

Pregnancy

Genital HSV infection in pregnant women is not uncommon, but it must be considered separately from nonpregnant populations because of the risk to the fetus and newborn. Recurrent infection is the most common form of infection during pregnancy. Transmission of infection to the fetus is related to shedding of virus at the time of delivery. The prevalence of viral excretion at delivery, as determined by culture, is 0.01–0.39% for all women, irrespective of past history (87), with rates as high as 0.6% (103). In a predominantly white, middle-class population, documented recurrent infection occurs in 84% of pregnant women with a history of recurrent disease. Asymptomatic viral shedding occurred in at least 12% of the recurrent episodes. Viral shedding from the cervix has occurred in 0.56% of symptomatic infections versus 0.66% of asymptomatic infections (87). The prevalence of cervical shedding in pregnant women with asymptomatic HSV infection averages approximately 3%. However, the observed rate for these women varies more than that among nonpregnant women (from 0.2% to 7.4%), depending upon the study population and trial design (87, 104).

PATHOGENESIS

Pathology

The histopathologic characteristics of a primary and recurrent HSV infection (Fig. 5) reflect virus-mediated cellular death with an associated inflammatory response. Viral infection induces ballooning of cells with condensed chromatin within the nuclei of cells, followed by nuclear degeneration, generally within parabasal and intermediate

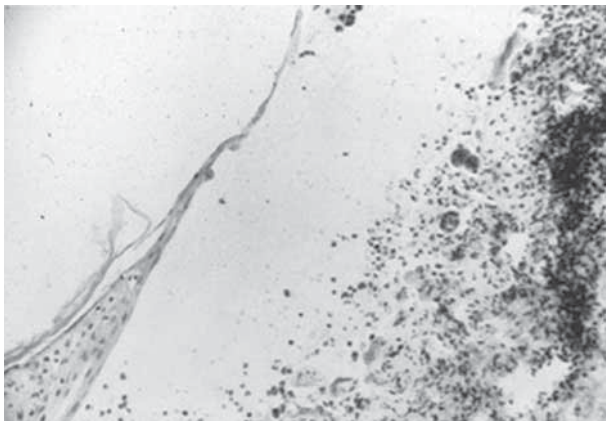

FIGURE 5 Histopathology of herpes simplex virus infection.

cells of the epithelium. Cells lose intact plasma membranes and form multinucleated giant cells. With cell lysis, a clear (referred to as vesicular) fluid containing virus appears between the epidermis and dermal layer. The vesicular fluid contains cell debris, inflammatory cells, and often multinucleated giant cells. In dermal substructures, there is an intense inflammatory response, usually in the corium of the skin, more so with primary infection than with recurrent infection. With healing, the vesicular fluid becomes pustular with the recruitment of inflammatory cells and scabs. Scarring is uncommon. When mucous membranes are involved, vesicles are replaced by shallow ulcers.

Pathogenesis

The transmission of human infection is dependent upon intimate, personal contact between a susceptible seronegative individual with someone excreting HSV. Virus must come in contact with mucosal surfaces or abraded skin for infection to be initiated. Following viral replication at the site of infection, either an intact virion, or more simply, the capsid is transported retrogradely by neurons to the dorsal root ganglia where, after another round of viral replication, latency is established (Fig. 6). The more severe the primary infection, as reflected by the size, number, and extent of lesions, the more likely it is that recurrences will ensue. Although replication sometimes leads to disease and infrequently results in life-threatening infection (e.g., encephalitis), the host–virus interaction leading to latency predominates, as noted above. After latency is established, a provocative stimulus causes reactivation; virus becomes detectable at mucocutaneous sites, appearing as skin vesicles or mucosal ulcers (Fig. 7).

Infection with HSV-1 generally occurs in the oropharyngeal mucosa. The trigeminal ganglion becomes colonized and harbors latent virus. Acquisition of HSV-2 infection is usually the consequence of transmission by genital contact; however, genital–oral spread is increasingly common and results in alternative modes of transmission from mucous membrane to mucous membrane for both viruses. Virus replicates in the genital, perigenital, or anal skin sites with seeding of the sacral ganglia (Fig. 8).

Operative definitions of the nature of the infection are of pathogenic relevance. Susceptible individuals (namely, those without preexisting HSV antibodies) develop primary infection after the first exposure to either HSV-1 or HSV-2. A recurrence of HSV is known as recurrent infection. Initial infection is when an individual with preexisting antibodies to one type of HSV (namely, HSV-1 or HSV-2) can experience a first infection with the opposite virus type (namely, HSV-2 or HSV-1, respectively). Reinfection with a different strain of HSV can occur, although it is extremely uncommon in the normal host, and is called *exogenous reinfection*.

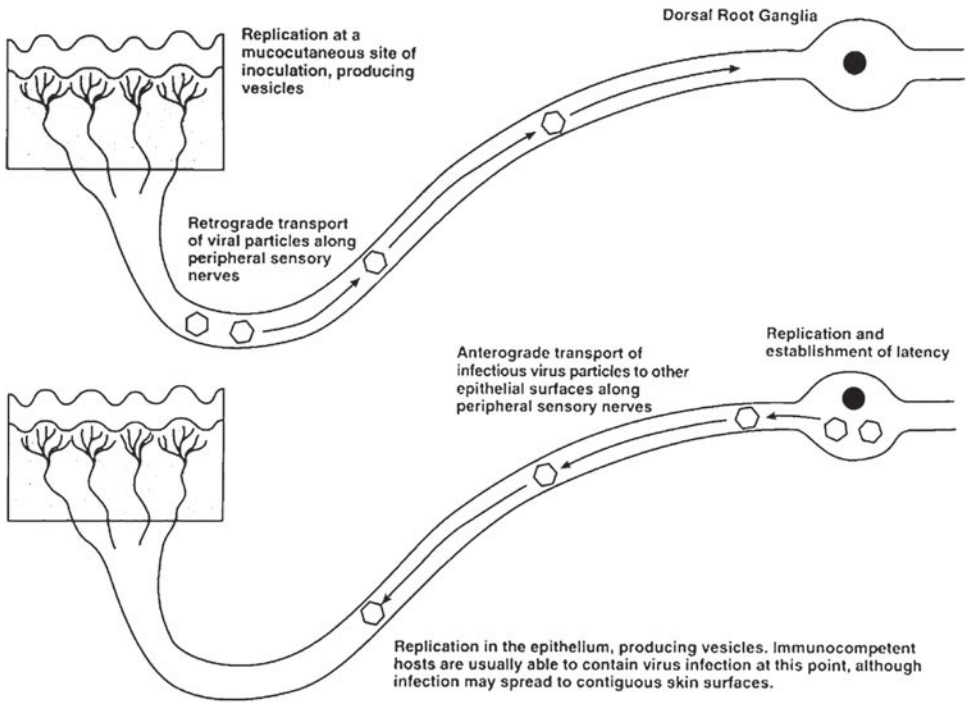

FIGURE 6 Schematic diagram of herpes simplex virus (HSV) infection.

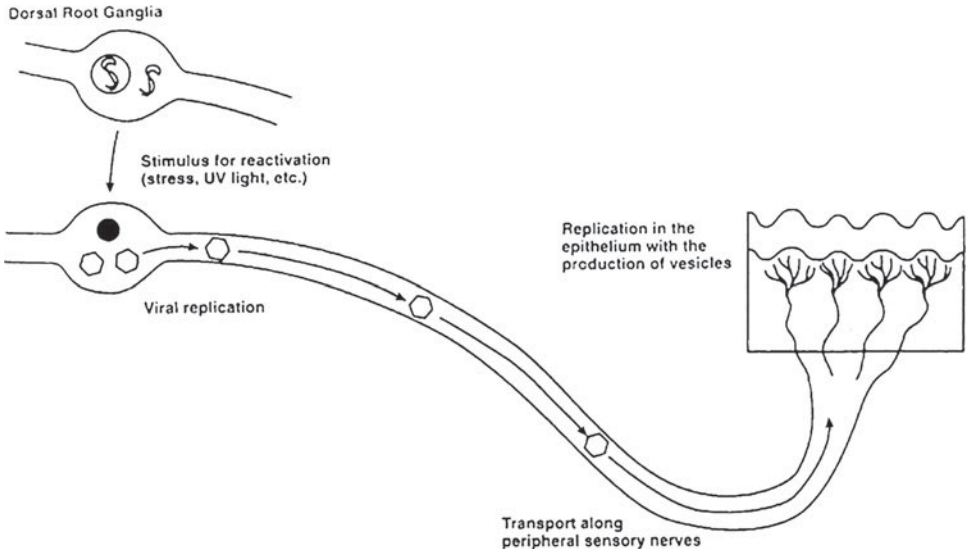

FIGURE 7 Schematic diagram of latency and reactivation.

Cleavage of HSV DNA by restriction endonuclease enzymes yields a characteristic pattern of subgenomic products. Analyses of numerous HSV-1 and HSV-2 isolates from a variety of clinical situations and widely divergent geographic area demonstrate that epidemiologically unrelated strains yield distinct HSV DNA fragment patterns. In contrast, fragments of HSV DNA derived from the same individual obtained years apart, from monogamous sexual partners, or following short and long passages *in vitro*, have identical fragments after restriction endonuclease cleavage (105). Utilizing endonuclease technology, exogenous reinfection is exceedingly low in the immune competent host.

Immune Responses

Primary HSV Infection

The host immune response to HSV infection has been recently reviewed (106). Following primary HSV infection, the initial, local immunological responses involve both nonspecific defense mechanisms, namely IFN-α and IFN-β, activated natural killer (NK) cells and macrophages, as well as HSV-specific responses, such as cytotoxic T cells (CTLs) (107) (see Chapter 16). The initial cellular response is synthesis and secretion of type I IFNs (α and β), leading to IFN-mediated activation of cellular enzymes such as 2'-5'-oligoadenylate synthetase (2'-5' AS) and dsRNA-dependent protein kinase, as well as intracellular signaling molecules through the activation of the Janus kinase (JAK)/STAT pathway. Relevant to HSV infection, IFN-α appears to inhibit IE gene expression (108). Thus, the antiviral mechanism directly affects transactivation of the IE responsive element necessary for synthesis of viral proteins.

IFNs also mediate macrophage and NK cell activation, activate CTLs, and induce major histocompatibility complex (MHC) class I and MHC class II antigens. In addition, cytokine secretion is stimulated, resulting in local inflammation. The role of IFN-γ is less clear, although γδ T cells, NK cells, $CD4^+$ T cells, and possibly neurons produce IFN-γ

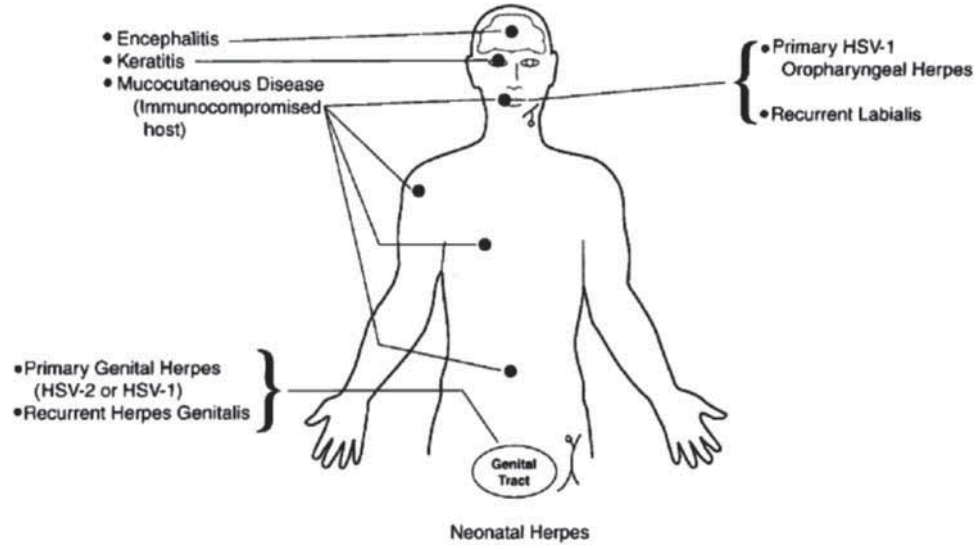

FIGURE 8 Sites of herpes simplex virus (HSV) infection and disease.

and TNF-α in response to HSV infection. IFN-γ downregulates priming of $CD4^+$ Th2 cells, which are responsible for inducing immunoglobulin (Ig) isotype B cell switching from IgA to IgG, thereby effecting humoral immune responses (109).

In vitro and *in vivo* experiments have demonstrated that NK cells protect from HSV challenge in a murine model (110). Severe herpetic disease has been correlated with low *in vitro* NK activity in newborns, as well as in a patient lacking NK cells (111). Other mononuclear cells, such as macrophages, are recruited to the site of infection, and upon activation, release immune cell mediators such as TNF and interleukins. Macrophages play a major role in mediating antibody-dependent cellular toxicity for viral clearance and antigen presentation (112).

As reviewed (106), an important aspect of immune responses to HSV infections is the maturation of dendritic cells (DCs) at the site of infection. Mobile DCs travel from mucosal or skin areas of infection and prime antigen-specific, naïve T-cells in draining lymph nodes (DLNs). In HSV-1 footpad infection of mice, fractionation of DC subpopulations in the DLN shows that classic $CD8\alpha^+$ dermal DCs, rather than specialized epidermal Langerhan's cells (LCs), prime naïve $CD8^+$ T cells (113). In the case of vaginal HSV-2 infection, dermal DCs rather than LCs again seem to be the physiologically active cell population in a similar DLN model (114). LCs are certainly able to present HSV antigens to memory HSV-specific T cells and may participate in primary or recurrent immune reactions. Plasmacytoid DCs (pDCs) react to HSV and produce IFN-α (115). pDCs are recruited to sites of infection, participate in viral clearance, and express relevant Toll-like receptors (TLRs), including TLR 7, 8, and 9, all of which recognize HSV (116, 117). Of interest, a low number pDCs or poor pDC reactivity is associated with severe human HSV infection (118, 119).

As infection progresses, virus-specific immune responses are detected. On days 4 and 5 postinfection, HSV-specific $CD4^+$ Th1 lymphocytes are detected in genital lymph nodes and in smaller numbers in the peripheral blood; they can subsequently be found in the genital mucosa (120). CD8 responses also occur quite quickly in the mouse (121); relevant human studies have not yet been reported.

Humoral immune responses rapidly follow initial HSV infection (106). The predominant mucosal antibodies are of the IgA isotype, which can be detected as early as day 3 following infection, peaking within the first 6 weeks after disease onset, and are followed by appearance of IgG1 and IgG3 subclasses of antibodies. HSV-specific IgA antibodies are present for at least 6 weeks, gradually decreasing to undetectable levels. IgM-secreting B cells have also been detected in secretions of the female genital mucosa (122). Shorter periods of viral shedding in women with primary genital herpes have been positively correlated with presence of secretory IgA in vaginal secretions (110).

Recurrent Infections

Although immunosuppression enhances the frequency of reactivation, there is no proof that the immune system exerts any influence on reactivation at the level of the ganglia (123). Immunosuppression enhances the detection of HSV reactivation in the periphery. It is not clear if this is due to increased ganglionic reactivation or failure of control mechanisms in the skin to contain virus being delivered down the axon. HSV-specific CD8 and CD4 T cells persistently infiltrate latently infected trigeminal ganglia in mice and humans (124, 125). These cells appear to act via IFN-γ (126). They have cytolytic activity, but neuron loss is not seen clinically, and inhibitory receptor-ligand pairings can be documented in the ganglia that may modulate their cytotoxic activity (127). In the periphery, HSV-2-specific CD4 and CD8 T cells localize to sites of recurrent HSV-2 infection and to the cervix (128, 129). Using *in situ* staining, HSV-2-specific CD8 CTLs have been shown to persist at the epidermal/dermal junction adjacent to sensory nerve endings (130). Damage to these cell populations in immunocompromised persons may lead to increased HSV replication either centrally or peripherally (112). Repeated subclinical episodes of HSV excretion may be a source of antigenic stimulation, leading to long-term HSV-specific immune memory (112). With recurrent HSV-2 infections, NK and HSV-specific $CD4^+$ cells are detected earlier than $CD8^+$ cells in genital lesions (109). CD4+ T cells and, more recently, $CD8^+$ T cells have been highlighted as major mediators of viral clearance from mucocutaneous lesions in recurrent episodes (107, 128). Low IFN-γ titers in vesicular fluid have been associated with a shorter time to the next recurrence in patients with frequent recurrences. T-cell proliferation is decreased in these patients in comparison to patients with less frequent recurrences (112). Inasmuch as the involvement of cytokines has been studied, IFN-γ has been reported to have a role in viral clearance from mucocutaneous sites, whereas altered cytokine production appears to correlate with recurrence (131).

As with primary HSV infection, a shorter duration of viral shedding occurs in women with recurrent genital herpes who have detectable secretory IgA in vaginal secretions (110). IgA, IgG1, and IgG3 antibodies have been found in the sera of all patients with recurrent HSV-2 episodes, whereas IgM and IgG4 antibodies were detected in 70–80% of these patients. However, there does not appear to be clear correlation between humoral immune responses and disease prognosis (132).

Newborn Infections

Host response of the newborn to HSV must be defined separately from that of older individuals, in part because of the immaturity of their host defense mechanisms. Other factors in defining host response of the newborn include the mode of transmission of the agent (viremia versus mucocutaneous infection without bloodborne spread), time of acquisition of infection, and the potential of increased virulence of certain strains, although this last point remains purely speculative. Two broad issues are of particular relevance in newborns. First, transplacentally acquired neutralizing antibodies and those mediating antibody-dependent cell-mediated cytotoxicity (ADCC) can either prevent or ameliorate infection in exposed newborns (133–135). Importantly, preexisting antibodies, indicative of prior infection, significantly decrease the transmission of infection from pregnant women to their offspring (57), contributing to the rationale for the development of a HSV vaccine.

Humoral IgG and IgM responses have been well characterized. Infected newborns produce IgM antibodies specific for HSV, as detected by immunofluorescence, within the first 3 weeks of infection. These antibodies increase rapidly in titer during the first 2–3 months, and they may be detectable for as long as 1 year after infection. The most reactive immunodeterminants are the surface viral glycoproteins, particularly gD. Humoral antibody responses have been studied using contemporary immunoblot technology, and the patterns of response are similar to those encountered in adults with primary infection (71, 136). The quantity of

neutralizing antibodies is lower in babies with disseminated infection (71, 136).

Second is the nature of immune responses mounted by HSV-infected newborns. The T-lymphocyte proliferative response to HSV infections is delayed in newborns compared to older individuals (71). Most infants have no detectable T-lymphocyte responses to HSV 2–4 weeks after the onset of clinical symptoms (71, 74, 137). The correlation between these delayed responses may be of significance in evaluating outcome to neonatal HSV infection. Specifically, if the response to T-lymphocyte antigens in children who have disease localized to the skin, eye, or mouth at the onset of disease is significantly delayed, disease progression may occur at a much higher frequency than in babies with a more appropriate response (71, 138).

Infected newborns have decreased production of IFN-α in response to HSV compared to adults with primary HSV infection (71). The importance of the IFN generation on the maturation of host responses, particularly the elicitation of NK cell responses, remains to be defined (139, 140). Lymphocytes from infected babies have decreased responses to IFN-γ during the first month of life (71, 140, 141). These data taken together would indicate that the newborn has a poorer immune response than older children and adults. Antibodies plus complement and antibodies mixed with killer lymphocytes, monocytes, macrophages, or polymorphonuclear leukocytes will lyse HSV infected cells *in vitro* (94). ADCC is an important component of the development of host immunity to infection (88). However, the total population of killer lymphocytes of the newborn seems to be lower than that found in older individuals, and monocytes and macrophages of newborns are not as active as those of adults (3,142–145).

CLINICAL MANIFESTATIONS

Oropharyngeal Disease

Primary Infections

Great variability exists in the symptoms of primary HSV-1 infections. Asymptomatic infection is the rule rather than the exception and occurs twice as often as symptomatic infection. Manifestations of disease range from totally asymptomatic to combinations of fever, sore throat, ulcerative and vesicular lesions, gingivostomatitis, edema, localized lymphadenopathy, anorexia, and malaise. The incubation period ranges from 2 to 12 days with a mean of approximately 4 days. In a study of 70 children with serologically documented primary infection, only six (less than 10%) had clinical symptoms associated with infection (147). In an Australian study, 67.4% of seronegative children (29 of 43) developed HSV antibodies over a period of 1 year; 69% had asymptomatic infection. Importantly, asymptomatic infection occurs twice as often as symptomatic disease.

Primary HSV-1 infection results in oral shedding of virus in mouth for as long as 23 days (average of 7–10 days). The natural history of HSV-2 infection of the oropharynx is not well characterized. Neutralizing antibodies appear between days 4 and 7 after onset of disease and peak in approximately 3 weeks (5, 6).

Symptomatic disease in children is characterized by involvement of the buccal and gingival mucosa (Fig. 9). The duration of illness is 2–3 weeks, with fever ranging between 101°F and 104°F. Often, children with symptomatic primary infection are unable to take liquids orally because of the associated pain. Lesions within the mouth evolve from vesicles to shallow ulcerations on an erythematous base before healing. Submandibular lymphadenopathy is common with primary gingivostomatitis but rare with recurrent infections. Other findings include sore throat and mouth, malaise, tender cervical lymphadenopathy, and an inability to eat. A clinical distinction should be drawn between intraoral gingival lesions and lip lesions indicative of presumed primary and recurrent infections, respectively.

FIGURE 9 Herpes simplex gingivostomatitis.

Primary HSV infections of adolescents and adults cause both pharyngitis and a mononucleosis syndrome (67). The differential diagnosis of both primary HSV gingivostomatitis and pharyngitis includes herpangina (usually caused by the coxsackieviruses), candidal infections of the mouth, Epstein-Barr virus-induced mononucleosis, lesions induced by chemotherapy or radiation therapy, and Stevens-Johnson syndrome.

Recurrent Infections

Recurrent infection may be asymptomatic, occurring in about 1% in children and 1–5% in immunocompetent adults (67, 147, 148, 150, 151). Nearly 1% of pregnant women and nursery personnel excrete HSV at any time (151), being a source of virus for transmission to the newborn. Asymptomatic excretion of virus is not limited to the healthy adult, as excretion of HSV in renal transplant recipients without signs or symptoms of disease occurs in nearly one-third of seropositive patients (152).

The onset of recurrent orolabial lesions is heralded by a prodrome of pain, burning, tingling, or itching, which generally lasts for 6 hours followed by the appearance of vesicles (149, 153). Vesicles appear most commonly at the vermillion border of the lip and persist for only 48 hours on the average (Fig. 10). Vesicles generally number three to five. The total area of involvement usually is localized, and lesions progress to the pustular or ulcerative and crusting stage within 72–96 hours. Pain is most severe at the outset and resolves quickly over 96–120 hours. Similarly, the loss of virus from lesions decreases with progressive healing over 2–3 days (66, 153). Healing is rapid, generally being complete in 8–10 days. The frequency of recurrences varies among individuals (153).

Genital Disease

Primary and Initial Infections

Primary genital herpes manifests with macules and papules, followed by vesicles, pustules, and ulcers. Lesions persist

FIGURE 10 Recurrent herpes simplex labialis.

about 3 weeks (76, 154, 155). Primary infection is associated with larger quantities of virus replicating in the genital tract ($>10^6$ viral particles/0.2 ml of inoculum) and a period of viral excretion, which may persist for 3 weeks. Systemic complications in the male are relatively uncommon; however, aseptic meningitis can develop. Paresthesias and dysesthesias which involve the lower extremities and perineum can result from genital herpetic infection.

Primary infections are usually associated with fever, dysuria, localized inguinal adenopathy, and malaise in both men and women. The severity of primary infection and its association with complications are statistically higher in women than in men (76, 156). Systemic complaints are common in both sexes, approaching 70% of all cases. The most common complications include aseptic meningitis and extragenital lesions.

In women with primary infection, lesions appear on the vulva and are usually bilateral, as shown in Fig. 11, with the cervix invariably involved. The actual frequency of primary cervical infection in the absence of vulvar infection is unknown. Lesions usually are excruciatingly painful, associated with inguinal adenopathy and dysuria, and may involve the vulva, perineum, buttocks, cervix, and vagina. A urinary retention syndrome occurs in 10–15% of female patients, and as many as 25% of women will develop aseptic meningitis.

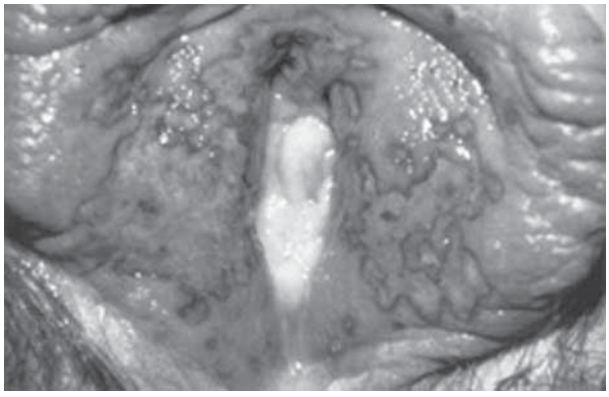

FIGURE 11 Genital herpes simplex virus infection (female).

In males, primary genital HSV infections are most often associated with vesicular lesions superimposed upon an erythematous base, usually appearing on the glans penis or the penile shaft, as shown in Fig. 12. The total number of lesions can vary significantly from 6 to 10 to many more. Extragenital lesions of the thigh, buttocks, and perineum can occur.

Complications following primary genital herpetic infection have included sacral radioculomyelitis, which can lead to urinary retention, neuralgias, and meningoencephalitis. Paresthesias and dysesthesias, which involve the lower extremities and perineum, may occur. Primary perianal and anal HSV-2 infections, as well as associated proctitis, are more common in male homosexuals. As with HSV-1 infections, as many as two-thirds of HSV-2 infections are subclinical, involving the mouth (73) or the uterine cervix (75, 92, 93). Nonprimary initial genital infection is less severe symptomatically, and heals more rapidly. The duration of infection is usually 2 weeks. The number of lesions, severity of pain, and likelihood of complications are significantly decreased. Preexisting antibodies to HSV-1 have an ameliorative effect on disease severity of HSV-2 (89).

Recurrent Infections

With recurrent genital herpetic infection, a limited number of vesicles, usually three to five, appear on the shaft of the penis of the male or as simply a vulvar irritation in the female. The duration of disease parallels that encountered with recurrent HSV labialis, being approximately 7–10 days. Neurologic or systemic complications are uncommon with recurrent disease; however, paresthesias and dysesthesias occur. Virus is shed for an average of 2–5 days and at lower concentrations (approximately 10^2–10^3/0.2 ml of inoculum in tissue culture systems) in women with recurrent genital infection. Recurrent genital herpetic infection in both men and women is characterized by prodrome, which is a useful marker for therapeutic trials and by localized irritation.

The frequency of recurrences varies from one individual to the next. Recurrences are more frequent in the first year after primary infection, and the severity of primary infection appears to correlate positively with the likelihood and

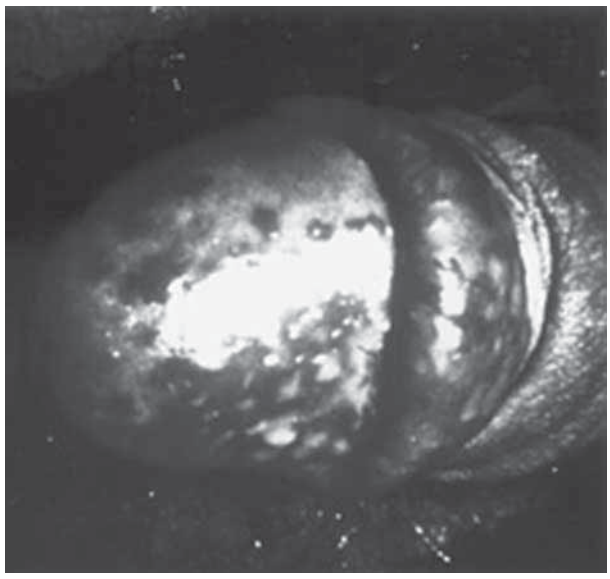

FIGURE 12 Genital herpes simplex virus infection (male).

frequency of symptomatic recurrences. One-third of infected individuals are estimated to have clinical recurrences in excess of eight or nine per year, one-third will have two to three per year, and the remaining one-third will have between four and seven (76). Individuals can transmit infection to sexual partners with symptomatic or, more commonly, asymptomatic recurrences. A high frequency of HSV DNA is detected by PCR in genital secretions between clinical recurrences (157). Many virologic recurrences may only be hours in duration (158). The implications of these data require the education of patients, including behavioral interventions and use of condoms to prevent transmission of infection.

Pregnancy and Neonatal Infection

An uncommon problem encountered with HSV infections during pregnancy is that of widely disseminated disease in the mother (159), involving multiple visceral sites and sometimes cutaneous dissemination. Dissemination after primary oropharyngeal or genital infection has led to necrotizing hepatitis with or without thrombocytopenia, disseminated intravascular coagulopathy, and encephalitis. The mortality rate among affected pregnant women is reported to be greater than 50%. Fetal deaths have also occurred in more than 50% of cases, although mortality did not necessarily correlate with the death of the mother. Factors associated with pregnancy may place both mother and fetus at increased risk for severe infection, possibly because of altered cell-mediated immunity.

The major risk to the fetus is primary or initial genital HSV infection of the mother during the third trimester of gestation (93, 103). Thus, identification of the woman at risk for primary infection is of paramount importance. Serologic discordance averages 15–20% such that the mother is HSV-2 seronegative and her partner is HSV-2 seropositive. The associated risk for transmission from the father is 10–15% during the pregnancy.

Maternal primary infection prior to 20 weeks gestation in some women has been associated with spontaneous abortion (160) in about 20% of pregnancies. The contribution of primary maternal genital infection to spontaneous abortion must be weighed above that of a routine rate of fetal loss of approximately 20%. Infection that develops later in gestation has not been associated with the termination of pregnancy, but infection of the fetus during maternal primary infection may result in manifestations of neonatal HSV disease, severe intrauterine growth retardation, or premature termination of gestation (161).

Neonatal Disease

The estimated incidence of neonatal HSV is approximately one in 2000 to one in 5000 live births annually in the United States. An increase in the number of cases of neonatal HSV infection has been noted in some areas, with rates approaching one in 1500 deliveries. Four factors influence the risk of HSV transmission of infection from mother to fetus. First the risk of transmission is 30–50% with maternal primary or initial infection as compared to 3% or less with recurrent infection (76, 103, 154). Second, paralleling the type of maternal infection, the mother's antibody status before delivery influences both the severity of infection and the likelihood of transmission. Transplacental maternal neutralizing and ADCC-mediating antibodies have at least an ameliorative effect on acquisition of infection for babies inadvertently exposed to virus at delivery (162). Third, prolonged rupture of membranes (greater than 6 hours) increases the risk of HSV acquisition as a consequence of ascending infection from the cervix. Fourth, fetal scalp monitors can be a site of inoculation of virus. Such devices should be relatively contraindicated in women with a history of recurrent genital HSV infection.

Infection of the newborn can be acquired at one of three times, and in all cases, the mother is the most common source of infection. First, *in utero* infection is rare and requires (163) stringent diagnostic criteria (namely, identification of infected babies within the first 48 hours of life who have virologic confirmation of infection). The second route of infection is that of intrapartum contact of the fetus with infected maternal genital secretions. Approximately 75–80% of babies acquire HSV infection by this route. The third route of transmission is postnatal acquisition. Although HSV-1 is associated with genital lesions, postnatal transmission of HSV-1 is increasingly suggested, with 15–20% of neonatal HSV infections caused by this type (3). Relatives and hospital personnel with orolabial herpes can be a reservoir for HSV infection of the newborn. The documentation of postnatal transmission of HSV has focused attention on such sources of virus for neonatal infection (164). Identical isolates, as demonstrated by restriction endonuclease technology, from babies born to different mothers in a nursery is rare. Postnatal transmission from mother to child has been documented as a consequence of nursing on an infected breast. Father-to-baby transmission has been documented from lesions of herpes labialis.

Clinical Presentation

Neonatal HSV infection is almost invariably symptomatic and frequently lethal. Babies with congenital infection should be identified within 48 hours following birth. Those babies who are infected intrapartum or postnatally with HSV infection can be divided into three categories—those with (118) disease localized to the skin, eye, and mouth (SEM) (165); encephalitis with or without skin involvement (40); and disseminated infection that involves multiple organs, including CNS, lung, liver, adrenals, skin, eye, and/or mouth (166).

Intrauterine Infection

Intrauterine infection is apparent at birth and is characterized by the triad of skin vesicles or skin scarring, eye disease, and the far more severe manifestations of microcephaly or hydranencephaly. Often, retinitis alone or in combination with other eye findings, such as keratoconjunctivitis, is a component of the clinical presentation. The frequency of occurrence of these manifestations is estimated to be one in 200,000 deliveries.

Disseminated Infection

Babies with the highest mortality present for therapy between 9 and 11 days of age. However, signs of infection usually begin on an average of 4–5 days earlier. With the early introduction of antiviral therapy, about 23% of babies with neonatal HSV infection have disseminated disease. The principal organs involved are the liver, lungs, and adrenals, but other involved organs include the larynx, trachea, esophagus, stomach, gastrointestinal tract, spleen, kidneys, pancreas, and heart. Constitutional signs and symptoms include irritability, seizures, respiratory distress, jaundice, bleeding diatheses, shock, and, frequently, the characteristic vesicular exanthem, which is often considered pathognomonic for infection. Encephalitis appears to be a common component of disseminated infection, occurring in about 60–75% of children. The vesicular rash, as described

below, is particularly important in the diagnosis of HSV infection. However, over 20% of these children do not develop skin vesicles during the course of illness (3, 167). Mortality in the absence of therapy exceeds 80%; all but a few survivors are impaired. The most common cause of death in babies with disseminated disease is either HSV pneumonitis or disseminated intravascular coagulopathy.

Encephalitis

Nearly one-third of all babies with neonatal HSV infection have encephalitis. Babies with infection of the CNS alone or in combination with disseminated disease present with the findings indicative of encephalitis in the newborn. Brain infection occurs either as a component of multiorgan disseminated infection or only as encephalitis with or without SEM involvement. The pathogenesis of these two forms of brain infection is likely different. Babies with disseminated infection probably seed the brain by a bloodborne route, resulting in multiple areas of cortical hemorrhagic necrosis. In contrast, babies who present with only encephalitis likely develop brain disease as a consequence of retrograde axonal transport of virus to the CNS. Two pieces of data support this contention. Babies with disseminated disease have documented viremia and are hospitalized earlier in life than those with only encephalitis, 9–10 days versus 16–17 days, respectively. Babies with encephalitis alone are more likely to receive transplacental neutralizing antibodies from their mothers, which may prevent viremia, allowing for only intraneuronal transmission of virus to the brain.

Clinical manifestations of either encephalitis (alone or in association with disseminated disease) include seizures (both focal and generalized), lethargy, irritability, tremors, poor feeding, temperature instability, bulging fontanelle, and pyramidal tract signs. Children with encephalitis without disseminated disease have skin vesicles in approximately 60% of cases at any time during the disease course (3, 167). Virus can be cultured from cerebrospinal fluid (CSF) in 25–40% of all cases. CSF findings usually include pleocytosis and proteinosis (as high as 500–1000 mg/dl), although a few babies with CNS infection have no CSF abnormalities. Death occurs in 50% of babies with CNS disease who are not treated and is usually related to brain stem involvement. With rare exceptions, survivors are left with severe neurologic impairment. The long-term prognosis following either disseminated infection or encephalitis is poor. As many as 50% of surviving children have some degree of psychomotor retardation, often in association with microcephaly, hydrancephaly, porencephalic cysts, spasticity, blindness, retinitis, or learning disabilities.

The diagnosis of encephalitis can be challenging. For children with disease localized to the CNS, skin vesicles, the classic sign of disease, may not be present in as many as 40% of babies. Thus, for the baby with CSF pleocytosis and protein elevation at 2–3 weeks of life, other diagnostic clues, such as skin vesicles, may not be present. For the neonate with CSF findings indicative of infection, HSV must be considered along with other bacterial pathogens (e.g., group B streptococcus, *Escherichia coli*, etc.). A reasonable diagnostic approach, if all antigen and Gram stain studies are negative, would be serial CSF examinations to document progression in protein and mononuclear cell counts.

Skin, Eye, and/or Mouth Infection

Infection localized to the SEM is associated with morbidity and not mortality. Vesicles occur in 90% of children with SEM infection. When infection is localized to the skin, the presence of discrete vesicles remains the hallmark of disease. Clusters of vesicles often appear initially on the part of the body that was in direct contact with the virus during birth. With time, the rash progresses to involve other areas of the body as well, particularly if viremia occurs. Vesicles occur in 90% of children with SEM infection. Children with disease localized to the SEM generally present at about 10–11 days of life. Babies with skin lesions invariably will suffer from recurrences over the first 6 months (and longer) of life, regardless of whether therapy was administered. Approximately 30% of these children eventually develop evidence of neurologic impairment in the absence of therapy (4). The skin vesicles (Fig. 13) usually erupt from an erythematous base and are usually 1–2 mm in diameter. Other manifestations of skin lesions include a zosteriform eruption. Infections involving the eye manifest as keratoconjunctivitis, or later, as retinitis. The eye can be the only site of HSV involvement in the newborn.

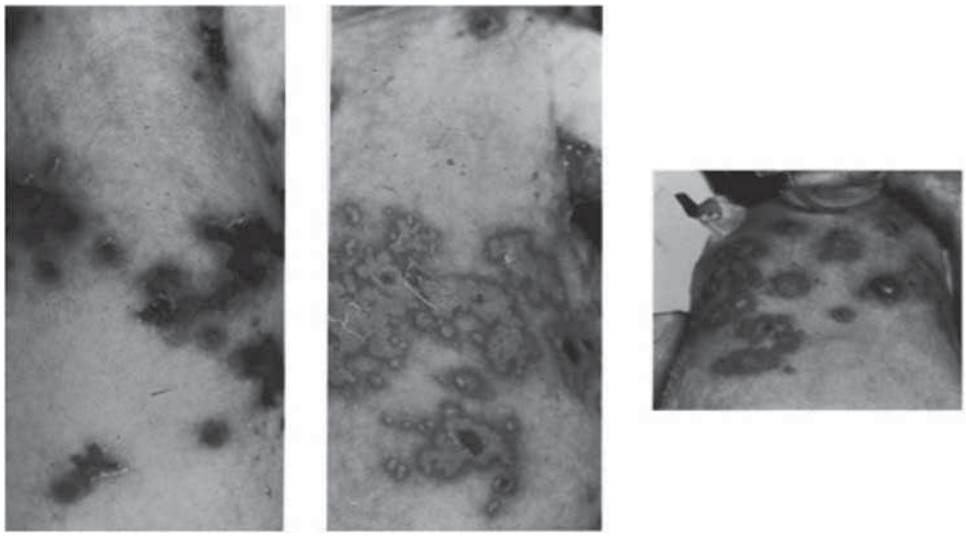

FIGURE 13 Vesicular rash of neonatal herpes simplex virus infection.

Long-term neurologic impairment may develop in children whose disease appears localized to the SEM. The significant findings include spastic quadriplegia, microcephaly, and blindness. Despite normal clinical examinations in these children, neurologic impairment has become apparent between 6 months and 1 year of life.

Prognostic Factors

Babies with the most severe disease have worse outcomes, as identified by disease classification (145). Level of consciousness also predicts a poor prognosis, as does the development of HSV pneumonia or disseminated intravascular coagulopathy. With encephalitis, prematurity and seizures predict a poor outcome. Finally, for babies with SEM disease, frequently recurrent HSV-2 cutaneous lesions define a group at risk for a poor neurologic outcome.

Application of PCR technology to CSF evaluation at the time of presentation SEM disease has identified a subgroup of babies with detectable HSV DNA. This group of children has asymptomatic infection of the CNS and accounts for those who develop neurologic impairment (168).

Herpes Simplex Keratoconjunctivitis

Viral infections of the eye beyond the newborn age are usually caused by HSV-1 (169). Approximately 300,000 cases of HSV infections of the eye are diagnosed annually in the United States where these infections are second only to trauma as the cause of corneal blindness. Herpetic keratoconjunctivitis is associated with either unilateral or bilateral conjunctivitis, which can be follicular in nature, followed soon thereafter by preauricular adenopathy. Eye infection is also associated with photophobia, tearing, eyelid edema, and chemosis, accompanied by the pathognomonic findings of branching dendritic lesions. Less commonly, with advanced disease, the infection is associated with a geographic ulcer of the cornea. Healing of the cornea can take as long as 1 month, even with appropriate antiviral therapy.

Recurrence frequency is similar to that for herpes labialis. Most frequently, lesions are unilateral in involvement, but a small percentage of cases involve both eyes. Characteristically, either dendritic ulceration or stromal involvement occurs. Visual acuity is decreased in the presence of the ulcers; and with progressive stromal involvement, opacification of the cornea may occur. Progressive disease can result in visual loss and even rupture of the globe.

Skin Infections

Infections of the digits, known as herpetic whitlow, are particularly common among medical and dental personnel. The estimated incidence is 2.4 cases per 100,000 population per year, being caused by HSV-1 or HSV-2. An increasing incidence of HSV-2 herpetic whitlow has been reported (170).

The prevalence of HSV skin infections in Skaraborg, Sweden, has been assessed among approximately 7500 individuals over 7 years of age and was has been found to be about 1% in those aged 7 years and older. In another Swedish study performed in dermatology clinics, 2% of men and 1.5% of women had evidence of herpetic skin infections over a 6-year period. In addition to individuals with atopic disease, patients with skin abrasions or burns appear particularly susceptible to HSV-1 and HSV-2 infections, and some develop disseminated infection (171).

Skin infections caused by HSV generally manifest as eczema herpeticum in patients with underlying atopic dermatitis, as reviewed in Chapter 8. Lesions can either be localized, resembling herpes zoster, or disseminated, as occurs with Kaposi's varicella-like eruption. Disseminated HSV infections have been reported among wrestlers (herpes gladitorium) (143). Burn patients are also at increased risk of severe skin infections, especially at sites of active epithelial regeneration. Other skin disorders associated with extensive cutaneous lesions include Darier's disease and Sezary's syndrome. As would be predicted, localized recurrences followed by a second episode of dissemination were observed. HSV infections of either type can trigger erythema multiforme (142). The detection of HSV DNA in skin lesions of erythema multiforme is as high as 80%.

Infections of the Immunocompromised Host

Patients compromised by immune therapy, underlying disease, or malnutrition are at increased risk for severe HSV infections. Renal, hepatic, bone marrow, and cardiac transplant recipients are all at high risk for increased severity of HSV infection (172) (Fig. 14). In organ transplant recipients, the presence of antibodies to HSV before treatment predicts the individual at greatest risk for recurrence (152). These patients may develop progressive disease involving the respiratory tract, esophagus, or even the gastrointestinal tract. The severe nature of progressive disease in these patients appears to be directly related to the degree of immunosuppressive therapy employed. Esophagitis is a common occurrence in the immunocompromised host and can be caused by HSV, CMV, or *Candida albicans*. Notably, acyclovir-resistant HSV disease can develop in treated immunocompromised hosts and be progressive. Reactivation of latent HSV infections in these patients can occur at multiple sites, and healing occurs over an average of 6 weeks (173).

Since the first reports of acquired immunodeficiency syndrome (AIDS), the increased frequency and severity of HSV clinical disease in these severely immunocompromised hosts was evident (174), especially in those with low CD4 counts. Because of persistent and high-level viral replication, resistance to antiviral therapy can develop. Asymptomatic excretion of HSV can occur even in the immunocompromised host. Parenthetically, acquisition of HSV infection from a transplanted organ (kidney) has been reported (175).

Infections of the CNS

Herpes simplex encephalitis is the most devastating of all HSV infections (Fig. 15) and is considered the most common

FIGURE 14 Cutaneous dissemination of herpes simplex virus infection in an immunosuppressed host.

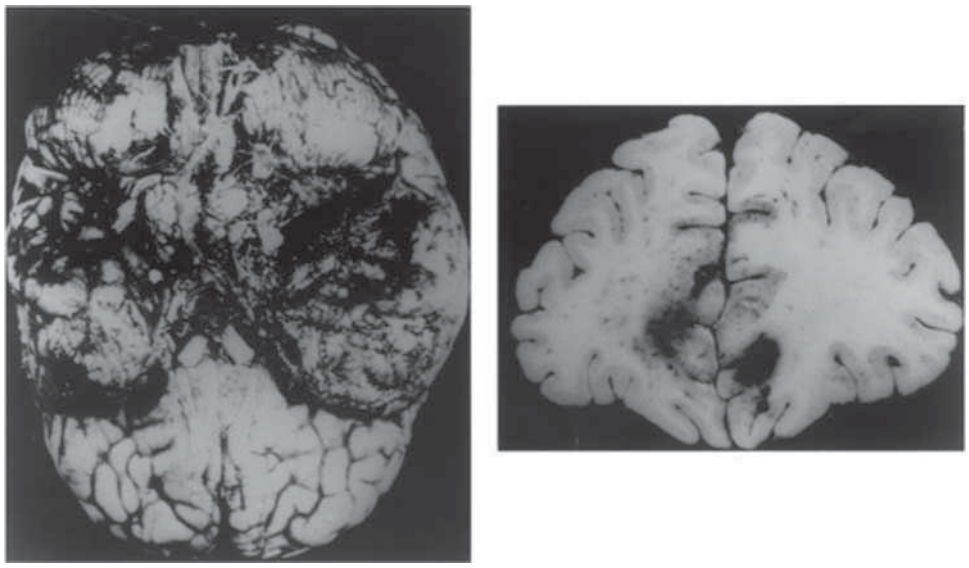

FIGURE 15 Coronal section of brain from patient with herpes simplex encephalitis.

cause of sporadic, fatal encephalitis (176). The incidence of severe hemorrhagic focal encephalitis is approximately one in 200,000 individuals per year, for a national annualized rate of approximately 1250 cases in the United States.

The manifestations of HSV encephalitis in the older child and adult are indicative of the areas of the brain affected, classically localized temporal lobe disease (166, 177). These include primarily focal encephalitis associated with fever, altered consciousness, bizarre behavior, disordered mentation, and localized neurologic findings. Clinical signs and symptoms reflect localized temporal lobe disease (166, 177). No signs are pathognomonic for HSV, but progressively deteriorating level of consciousness, expressive aphasia, bizarre behavior, fever, an abnormal CSF formula, and focal neurologic findings in the absence of other causes should make this disease highly suspect. Diagnostic evaluations should be initiated immediately, as other treatable diseases mimic HSV encephalitis. Mortality in untreated patients is in excess of 70%, and only 2.5% of untreated patients return to normal neurologic functions.

Standard neurodiagnostic procedures include CSF examination, electroencephalogram, and preferably a magnetic resonance image. Characteristic CSF abnormalities include pleocytosis (usually mononuclear) and elevated protein. Red blood cells are found in most (but not all) CSFs obtained from patients with HSV encephalitis. Upon serial examination, CSF protein levels and cell counts rise dramatically. The electroencephalogram generally localizes spike and slow-wave activity to the temporal lobe. A burst suppression pattern is characteristic of HSV encephalitis. Imaging will allow for localization of disease to the temporal lobe. Early after onset, only evidence of edema is detectable, if at all. This finding is followed by evidence of hemorrhage and midline shift in the cortical structures. Specific diagnostic assays are delineated below.

Other Neurologic Syndromes

In addition to encephalitis, HSV can involve virtually all anatomic areas of the nervous system, causing meningitis, myelitis, radiculitis, and other syndromes. Aseptic meningitis is a common occurrence in individuals with primary genital HSV infections.

Other Forms of Infection

HSV has been isolated from the respiratory tract of adults with adult respiratory distress syndrome and acute onset bronchospasm (139). Both were associated with increased mortality and morbidity.

Genetic Susceptibility

Genetic susceptibility to HSV infections is attracting increasing attention, as noted above with the CSSG-1. A growing body of evidence has defined a polymorphism resulting in an IFN-γ defect that has been reported in children with recurrent herpes encephalitis (178).

Furthermore, numerous reports have incriminated or refuted human leukocyte antigen (HLA) associations with human HSV infections. For recurrent fever blisters, these studies have included HLA-A1, HLA-A2, HLA-A9, HLA-BW16, and HLA-CW2. Recurrent ocular HSV infections have been associated with HLA-A1, HLA-A2, HLA-A9, and HLA-DR3 (44).

LABORATORY DIAGNOSIS

Polymerase Chain Reaction

The use of PCR is the diagnostic method of choice for HSV infections of the CNS (179, 180) and is rapidly replacing culture for routine skin and mucosal site infections because of its sensitivity. The sensitivity of PCR exceeds culture for detection of infection in genital and labial herpes (182).

Primers from an HSV DNA sequence common to both HSV-1 and HSV-2 (either the glycoprotein B domain or HSV DNA polymerase) can identify HSV DNA in CSF. The evaluation of CSF specimens obtained from patients with biopsy proven herpes simplex encephalitis (HSE) and those with proven other diseases indicates a sensitivity $>95\%$ at the time of clinical presentation and a specificity that approaches 100% (179, 181). False-negative assessments can be found when there is hemoglobin contamination in the CSF or the presence of inhibitors, such as heparin. Likely, PCR analyses of CSF specimens continue to reinforce the focal presentation of HSV infections of the CNS. Importantly, PCR evaluation of CSF can be used to

follow therapeutic outcome in patients with HSE. Persistence of HSV DNA in the CSF of newborns with suspected HSE requires continued therapy. The sensitivity of PCR exceeds culture for detection of infection in genital and labial herpes (182).

Cell Culture

Virus isolation remains an important diagnostic tool, especially if resistance is suspected. If skin lesions are present, a scraping of skin vesicles should be made and transferred in appropriate virus transport medium to a diagnostic virology laboratory. Clinical specimens should be shipped on ice for inoculation onto cell cultures (e.g., human foreskin fibroblasts, Vero cells, etc.), which are susceptible to cytopathic effects characteristic of HSV replication. Cytopathic effect usually develops within 24–48 hours after inoculation of specimens containing infectious virus, thus the shipping and processing of specimens should be expedited. In addition to skin vesicles, other sites from which virus may be isolated include the CSF, stool, urine, throat, nasopharynx, and conjunctivae. Duodenal aspirates from infants with hepatitis or other gastrointestinal abnormalities are useful for HSV isolation. Because outcome with treatment does not appear to be related to the virus type, identification is only of epidemiologic and pathogenetic importance, and, therefore, not usually necessary.

Cytologic Evaluation

Cytologic examination of cells from the maternal cervix or from the infant's skin, mouth, conjunctivae, or corneal lesions is of low sensitivity, being approximately 60–70%, and is not recommended (85). Cellular material obtained by scraping the periphery of the base of lesions should be smeared on a glass slide and fixed promptly in cold ethanol. The slide can be stained according to the methods of Papanicolaou, Giemsa, or Wright before examination by a trained cytologist. Giemsa or Tzanck smears likely will not demonstrate the presence of intranuclear inclusions. The presence of intranuclear inclusions and multinucleated giant cells are indicative, but not diagnostic, of HSV infection, as varicella-zoster virus induces the same histopathology. Electron microscopy assays are available but impractical.

Serologic Assessment

Serologic diagnosis of HSV infection is of little clinical value for purposes of therapeutic intervention but can assist in counseling sexually active individuals. Serologic assays that distinguish HSV-1 from HSV-2 are commercially available, being type specific. These tests are predicated on the use of gG-1 and gG-2 antigens and can assist physicians counseling individuals, i.e., discordant serologic status between sexual partners. The use of enzyme-linked immunoassay (ELISA) antibody assays only allows definition of past infection or recent seroconversion, but cannot distinguish HSV-1 from HSV-2. Other commonly used tests for measurement of HSV antibodies are complement fixation, passive hemagglutination, neutralization, and immunofluorescence.

PREVENTION

Education

Because of the increasing incidence of genital herpes and neonatal herpes and its association with acquisition of HIV, every effort should be made to prevent HSV-2 infections. Until a vaccine is proven effective, educational efforts must be developed for adolescents and adults at greatest risk. The use of condoms should be promoted.

Neonatal HSV Infection

The detection of type-specific antibodies to gG-2 will be of value in identifying those women at greatest risk. Documentation of discordant serologic status between sexual partners may assist in prevention of maternal primary infection. Clearly, male partners who are seropositive should be educated regarding HSV transmission.

Surgical abdominal delivery will decrease transmission of infection when membranes are ruptured less than 4 hours, but cesarean section has not been proven efficacious when membranes are ruptured for longer periods of time. Nevertheless, cesarean section is recommended when membranes are ruptured up to 24 hours in the presence of active lesions. Although the recommendation seems logical, no data exist to support it.

For women with a past history of genital HSV infection, a careful vaginal examination at presentation to the delivery suite is of paramount importance. Visualization of the cervix is often difficult, but speculum examination for documentation of recurrent lesions is important and should be attempted in all women. A culture for HSV obtained at the time of delivery can be of value in establishing whether excretion can lead to transmission of infection to the fetus. Clearly, identifying women who excrete HSV at delivery and then using either antiviral prophylaxis in the neonate with safe and acceptable antivirals or delivery by cesarean section remains the optimal management of genital infection at delivery.

For babies born to women with known primary infections during the third trimester, routine prophylaxis of the newborn with acyclovir is recommended by the American Academy of Pediatrics (183). On the other hand if the child is born in the presence of lesions, surface cultures are indicated and therapy initiated. If cultures are negative, treatment can be discontinued (183). An algorithm for managing these babies has been developed (184).

Nosocomial Transmission

At many institutions, a policy that requires transfer or provision of medical leave for nursery personnel who have a labial HSV infection is impractical and causes an excessive burden in those attempting to provide adequate care. Temporary removal of personnel who have cold sores has been suggested. Individuals with herpetic whitlows also shed virus, and these individuals should be removed from the care of newborns at risk for acquiring neonatal HSV infection because even gloves may not prevent transmission of infection. Education regarding the risks of transmission of virus and the importance of hand washing when lesions are present should be repeatedly emphasized to healthcare workers. In addition, hospital personnel should wear masks when active lesions are present.

VACCINE DEVELOPMENT

Development of an HSV vaccine has been attempted for over a century, but remains investigational at present. Two approaches have attracted the most attention, as reviewed (68, 106). The first is based on either microorganisms or cell lines producing gB or gD2 for use as subunit vaccines in combination with an adjuvant. The second is based on genetically engineering the virus to yield either a live, attenuated vaccine from which putative neurovirulence and immune evasion sequences have been removed or to produce a vaccine virus that is only capable of a single round of

replication. Each of these approaches has been evaluated extensively in animal models and to varying extents in human investigations. Extrapolating protection from animal model systems to humans has not been possible because there are no validated markers of protection comparable to neutralizing antibodies for other viral diseases (165,185–191). Initial HSV vaccines were oriented toward the prevention of recurrent infections and thus considered therapeutic vaccines, whereas more recent efforts have been devoted to the prevention of HSV infection or disease following exposure to an infected partner.

Subunit Vaccines

A history of HSV vaccines and basis for failure appears elsewhere (106, 192), and we will focus here on the current approaches. Subunit vaccines have been studied extensively, evolving from attempts to avoid viral DNA. In addition, these vaccines eliminate the potential for cellular transformation, enhance antigenic concentration, and induce stronger immunity as well as exclude any possibility of residual live virus contamination (193). Current subunit vaccines have been prepared by a variety of methods combining antigen extraction from infected cell lysates by detergent and subsequent purification; immunogenicity of vaccines derived from all of the envelope glycoproteins, free of viral DNA, has been demonstrated in animals (194–197). However, vaccination with envelope glycoproteins does not protect uninfected sexual partners against genital HSV infection (198, 199).

Specific subunit vaccines have been developed by cloning selected glycoproteins in either yeast or Chinese hamster ovary cell systems (186, 200, 201), as well as by other methods (202–204). When studied in a variety of animal models (205, 206), neutralizing antibodies can be detected in vaccinated animals, and in some systems, binding antibodies as well (207). In these systems the quantity of neutralizing antibody correlates with the degree of protection upon challenge. Because each of these models utilized different routes of challenge and different viral inocula, interpretation of these results is extremely difficult. In general, the subunit vaccines elicit a degree of protection, as evidenced by amelioration of morbidity and reduction in mortality in immunized animals. Several injections and an appropriate adjuvant are required to induce protection (208, 209). Notably, protection in the rodent is significantly easier than in primate species. This may be especially the case because the HSV is not indigenous to rodent species, and thus protection studies may be totally irrelevant when evaluating human responses. Vaccination of primates, specifically rhesus monkeys (205), chimpanzees (194, 205), and rhesus monkeys (210), induces neutralizing antibodies, leading to an amnestic response following subsequent immunization months later. The significance of the protection in these animals remains unclear for human prophylaxis.

Clinical trials have evaluated gB2 plus gD2 and gD2 alone subunit vaccines in humans. One of the earliest human vaccine studies was with a glycoprotein envelope subunit vaccine (198, 199, 210, 211). In sexual partners of patients known to have genital herpes, the number of individuals developing herpetic infection was nearly equal between placebo and vaccine recipients; thus, vaccination failed to provide any benefit at all. However, vaccine immunogenicity was poor because it induced ELISA antibody titers to HSV-2 glycoproteins D (gD2) and B (gB2) that were only 10% and 5%, respectively, of titers found in persons with recurrent genital HSV-2 infection (196).

Subsequent gB2 plus gD2 and gD2 alone subunit vaccines incorporated unique adjuvants, namely MF59, potent for Th-2 responses, and AS04 (50 μg of 3-*O*-desacyl-4′-monophosphoryl lipid A (212) and 500 μg of aluminum hydroxide). Both afforded a high level of protection from HSV disease in animal models (208, 213), and notably elicited higher neutralizing antibody and the total HSV antibody titer (as measured by ELISA) titers than following infection by wild-type virus. Furthermore, these antibody titers correlated with protection from disease (214, 215).

In Phase III studies, the combined antigen vaccine-induced antibody titers exceeded those found in individuals who had HSV-2 infection. However, the vaccine failed to provide significant long-term prevention of infection in susceptible sexual partners, although 50% reduction in the rate of infection among HSV seronegative women was present for the first 5 months (216). The overall efficacy of the vaccine for 1 year following a 6-month vaccination period was 9%. The vaccine had no apparent effect on the frequency of recurrences amongst vaccine recipients who became infected (217).

The monovalent gD2 vaccine (134) is a potent inducer of Th1 responses (218) and the vaccine induces robust humoral and cellular immune responses in vaccinees. The vaccine-induced titers of HSV gD-specific antibody are higher than those observed in patients who had sexually acquired genital HSV-2 infection (219). In controlled studies, women who were seronegative for both HSV-1 and HSV-2 were significantly protected from disease (72% efficacy; p=0.01–0.02), and there was a trend toward protection against infection (43% efficacy; p=0.06–0.07). However, in individuals seropositive for HSV-1, irrespective of sex, and seronegative men, no significant clinical benefit could be demonstrated. A Phase III study sponsored by the National Institutes of Health failed to protect against HSV-2 but did impact HSV-1. Likely the changing epidemiology of genital herpes, namely an increase in genital HSV-1 infection, influenced these results.

Live-Attenuated Vaccines

Live vaccines, in general, are considered more immunogenic, but have increased safety concerns compared to killed or subunit vaccines. Several approaches to live virus vaccines have been attempted: HSV mutants, heterologous herpesviruses, antigens expressed in non-HSV viral vectors, and genetically engineered viruses.

Numerous constructs have been tested in animal models, but none have advanced to Phase II or III trials (106, 192). The most promising are genetically engineered attenuated (220–223) HSVs. The construction of these viruses was based on the use of an HSV-1 [HSV-1(F)] as a backbone. The genome was deleted in the domain of the viral thymidine kinase (TK) gene and in the junction region between the unique long and short sequences to excise some of the genetic loci responsible for neurovirulence and to create convenient sites and space within the genome for insertion of other genes. Last, an HSV-2 DNA fragment encoding the HSV-2 glycoproteins D, G, and I was inserted in place of the internal inverted repeat. The purpose of type 2 genes was to broaden the spectrum of the immune response a chimeric pattern of antibody specificities as a serological marker of vaccination. Because TK is expressed, it is susceptible to antiviral chemotherapy with acyclovir. When evaluated in rodent models, pathogenicity was attenuated and the ability to establish latency diminished; however, protective immunity was not induced.

Similar results were found in owl monkeys (*Aotus trivirgatus*) (224). While 100 PFU of wild-type viruses administered by peripheral routes were fatal to the monkeys, recombinants given by various routes in amounts at least 10^5-fold greater were innocuous or produced mild infections, even in the presence of immunosuppression by total lymphoid irradiation (221).

Human studies with this vaccine were disappointing, as the maximum dose of vaccine administered (10^5 PFU) elicited only mild immunogenicity, even with the administration of two doses (224). The ability to pursue higher doses of vaccine was limited because of an inability to produce satisfactory concentrations of vaccine.

Another replication-impaired deletion mutant is soon approaching clinical trials, having shown promise in animal model systems (225, 226). This HSV vaccine is using replication-defective viruses, as reviewed (227). While first studied over a decade ago (228, 229), a current construct deletes U_L5 and U_L29 in HSV-2 to generate the dl5-29 vaccine candidate. Importantly, this vaccine candidate was compared to a gD2 subunit vaccine in Freund's adjuvant. Significant protection was induced along with high titers of neutralizing antibodies and $CD8^+$ T cells (230–232).

GENE THERAPY

The use of HSV for gene therapy has been reviewed (233). Genetically engineered HSV has undergone extensive preclinical studies and, recently, led to the licensure of one product for the treatment of nonsurgically resectable melanoma. Predicated upon the demonstration that viruses lacking the $\gamma_1$34.5 gene are incapable of replicating in postmitotic cells, such as the brain, an important degree of safety is incorporated; yet these viruses kill cancer cells. Furthermore, they can serve as a vector for foreign expression, particularly proinflammatory genes.

Melanoma

Indeed, an HSV deleted in $\gamma_1$34.5 and expressing granulocyte-macrophage colony-stimulating factor (GM-CSF), talimogene laherparepvec [IMLYGIC^R (Amgen)], was recently licensed in the United States and Europe for the treatment of stage IIIB, IIIC, and IV melanoma. The virus is derived from the pathogenic clinical isolate JS-1 (234). Attenuations in this vector include replacement of both $\gamma_1$34.5 genes with the gene encoding human GM-CSF and deletion of the α47 coding sequence such that the US11 gene is expressed from the IE α47 promoter. This virus is "armed," meaning it expresses a transgene hypothesized to enhance antitumor effects by complementing the lytic mechanism of tumor clearance. In this case, the additional mechanism is immune stimulation through the cytokine GM-CSF, a hematopoietic growth factor that promotes the maturation and development of DCs as well as eliciting the formation of Th1 skewed immune responses.

Glioblastoma Multiforme

Genetically engineered HSV has been assessed for the treatment of human glioblastoma multiforme. These constructs have included mutations in the viral genes TK, DNA polymerase, ribonucleotide reductase, and $\gamma_1$34 (235–241) and have been identified as HF10, HSV1716, NV1020, G207, G47Δ, OncoVEX$^{GM\text{-}CSF}$, rRp450, M032, and C134 (224, 234,242–245). While virtually any alteration of HSV ameliorates neurovirulence, only the deletions in the $\gamma_1$34.5 gene consistently demonstrate safety and efficacy in animal models. Tumoricidal effects are demonstrable *in vitro* and *in vivo* in multiple glioma models (mouse, rat, and human glioma cell lines, human glioma explants). *In vivo* models include tumor reduction in subrenal capsule and flank subcutaneous implants, but more importantly, increased survival and some tumor cures in intracranial implant models. These effects are reproducible *in vivo* for both immune-deficient animals (nude, *scid* mice) (236,239–241) as well as immune-competent models (rats and mice) (237, 238, 241, 246). Three approaches to the experimental therapy of glioblastoma are in human investigations.

G207

G207, derived from the HSV-1 (F) strain, is deleted for both $\gamma_1$34.5 genes and is further attenuated by insertion of the *lacZ* gene into the UL39 locus, preventing expression of ribonucleotide reductase. G207 was demonstrated to be safe following direct intracranial injection in both mice and in highly susceptible *Aotus nancymaae* primates (247, 248).

The first Phase I safety trial with G207 evaluated direct intratumoral injection of virus in escalating doses ranging from $1x10^6$ to $3x10^9$ PFU. A total of 21 patients with recurrent grade III–IV malignant glioma were enrolled in the trial (249). Even at the highest dose ($3x10^9$ PFU), no significant adverse events related to G207 administration were reported. Importantly, no evidence of encephalitis was apparent in any biopsy samples taken to monitor tumor progression or at postmortem.

Additional Phase Ib studies in patients with recurrent glioblastoma multiforme have employed a variety of designs to define safety upon injection into a resected tumor bed, the ability of the virus to replicate in tumors, the effect of multiple administrations, and the definition of the contribution of radiation to improved outcome (250). At total doses of $1.15x10^9$ PFU of G207, the results continue to define safety, evidence of virus replication at the highest doses, radiographic improvement, and some long-term survivors.

HSV1716

HSV1716 is one of two conditionally replication-competent, engineered HSVs directly injected into malignant glioma with demonstrated safety and the absence of neurovirulence. HSV1716 has been clinically studied for the treatment of malignant glioma in the United Kingdom. A series of Phase I studies of direct intratumoral and surrounding normal parenchyma administration of HSV1716 at doses ranging from $1x10^3$ to $1x10^5$ PFU proved safety and, subsequently, viral replication in the tumor (212, 251, 252).

HSV1716 has also been assessed as a therapy of oral squamous cell carcinoma ($1x10^5$ PFU of virus intratumorally) and melanoma with evidence of safety but no documentation of viral replication (253, 254).

M032, C134

The engineered HSV recombinants M032 and C134 are similar in that both were originally derived from the wild-type HSV-1 (F) strain. They share identical deletions of the $\gamma_1$34.5 gene, but differ with regard to the inserted genes replacing $\gamma_1$34.5. M032 expresses the human immunostimulatory cytokine IL-12. IL-12 promotes the activation of IFN-γ secretion and Th1 polarization of $CD8^+$ T lymphocytes and also has antiangiogenic effects. Preliminary studies of a murine IL-12-expressing virus M002 demonstrate increased survival in intracranial tumor-bearing mice treated with M002 and increased infiltration of innate and adaptive immune cells into tumors (255). An Investigational New

Drug (IND) application has been submitted to evaluate the safety of M032 in patients with recurrent glioblastoma and is currently awaiting FDA approval.

C134 expresses the CMV PKR evasion and IFN-γ resistance molecule IRS1 (256). IRS1 has equivalent functions as the $\gamma_1$34.5 gene product ICP34.5 in terms of PKR evasion, but unlike ICP34.5 it does not contribute to neurovirulence. Cells infected with C134 do not undergo PKR-mediated host protein shutoff. This has been found to occur in neuroblastoma cell lines, prompting the submission of an IND application for a Phase I clinical trial of C134 in pediatric patients with neuroblastoma (242). A recent review summarizes these data (257).

TREATMENT

Acyclovir has become the standard of systemic therapy of HSV infections. Acyclovir (9-[2-hydroxyethoxymethyl] guanine), its prodrug valacyclovir (Valtrex), and other antiherpes agents are described in detail in Chapter 12. When valacyclovir is administered at 1 gram every 8 hours, resulting plasma levels are similar to 5 mg/kg of acyclovir given intravenously. Famciclovir (Famvir), the prodrug of penciclovir, and valacyclovir are both available as generic drugs and provide enhanced oral bioavailability compared to acyclovir (81, 258, 259).

Genital Herpes

In initial genital HSV infection, topical application of acyclovir reduces the duration of viral shedding and the length of time before all lesions become crusted, but it is less effective than oral or intravenous therapy (260). Indeed, topical therapy is not recommended. Intravenous acyclovir is the most effective treatment for first-episode genital herpes and results in a significant reduction in the median duration of viral shedding, pain, and length of time to complete healing (8 versus 14 days) (261). Intravenous acyclovir therapy usually requires hospitalization, thus it should be reserved for patients with severe local disease or systemic complications. Oral acyclovir therapy (200 mg 5 times daily or 400 mg t.i.d.) is nearly as effective as intravenous acyclovir for initial genital herpes (156, 262), and was the first licensed approach. Because of ease of administration and improved pharmacodynamics, valacyclovir and famciclovir have replaced acyclovir at most institutions. Neither intravenous nor oral treatment of acute HSV infection reduces the frequency of recurrences (258, 261, 262).

Recurrent genital herpes is less severe and resolves more rapidly than primary infection; thus, there is less time to introduce antiviral chemotherapy successfully. Oral acyclovir therapy shortens both the duration of viral shedding and the length of time to healing (6 versus 7 days) when initiated early (within 24 hours of onset), but the duration of symptoms and length of time to recurrence are not affected (263, 264). Valacyclovir and famciclovir provide added benefit, particularly with short-course therapy as described in Chapter 12 (81, 258, 265, 266).

Long-term oral administration of acyclovir, valacyclovir, and famciclovir all effectively suppress genital herpes in patients who have frequent recurrences (137, 267, 268). Daily administration of acyclovir reduces the frequency of recurrences by up to 80%, and 25–30% of patients have no further recurrences while taking acyclovir (268). Successful suppression for as long as 10 years has been reported, with no evidence of significant adverse effects. Titration of the dose of acyclovir (400 mg twice daily or 200 mg two to five times daily) may be required to establish the minimal dose that is most effective and economical. The emergence of acyclovir-resistant strains of HSV appears to be infrequent in immunologically normal individuals (269). Importantly, asymptomatic shedding of virus can continue despite clinically effective suppression with acyclovir, so that the possibility of person-to-person transmission persists (136).

Valacyclovir and famciclovir are also efficacious in the treatment of recurrent genital herpes or for suppression. Valacyclovir for treatment is administered at a dosage of 500 mg b.i.d for 5–7 days and for suppression 500 mg or 1 gram daily. Famciclovir is administered at 250 mg t.i.d. for treatment and suppression. Valacyclovir was proven to decrease person-to-person transmission when administered to the seropositive partner (270).

Tenofovir topical gel has been shown to impact viral shedding but remains experimental at this time and may prove useful in the developing world because of its activity for preventing HIV transmission as well.

Herpes Labialis

Topical therapy is of no value. Orally administered acyclovir (200 mg 5 times or 400 mg t.i.d. daily for 5 days) reduces the length of time to the loss of crusts by approximately 1 day (7 versus 8 days), but does not alter the duration of pain or the length of time to complete healing (271). If the dose is increased to 400 mg 5 times daily for 5 days, treatment started during the prodromal or erythematous stages of infection reduces the duration of pain by 36% and the length of time to the loss of crusts by 27% (272). Thus, oral acyclovir has modest clinical benefit only if initiated very early after recurrence. Both valacyclovir and famciclovir can be administered for short courses, 3 days or 1 day, respectively, with similar results.

Oral administration of acyclovir can alter the severity of sun-induced labial reactivation of labial HSV infections. The administration of 200 mg five times daily to skiers did not decrease the frequency of recurrent labial infections as compared with placebo, but significantly fewer lesions formed on days 5–7 among acyclovir recipients. Short-term prophylactic therapy with acyclovir may benefit some patients with recurrent herpes labialis who anticipate engaging in a high-risk activity (e.g., intense exposure to sunlight). The intermittent administration of acyclovir does not alter the frequency of subsequent recurrences. No data support the use of long-term treatment with acyclovir for the prevention of herpes labialis.

Mucocutaneous HSV Infections in Immunocompromised Patients

HSV infections of the lip, mouth, skin, perianal area, or genitals may be much more severe in immunocompromised patients than in normal hosts. In the former, the lesions tend to be more invasive, slower to heal, and associated with prolonged viral shedding. Intravenous acyclovir therapy is beneficial in such patients (141). Immunocompromised patients receiving acyclovir had a shorter duration of viral shedding and more rapid healing of lesions than patients receiving placebo (273). Oral therapy with valacyclovir and famciclovir are also very effective in immunocompromised patients (274).

Antiviral prophylaxis of HSV infections is of clinical value in severely immunocompromised patients, especially those undergoing induction chemotherapy or transplantation. Intravenous of acyclovir or oral administration of any of the three drugs reduces the incidence of symptomatic

TABLE 1 Indications for acyclovir therapy

Type of infection	Route and dosage*	Comments
Genital HSV		
Initial episode	200 mg p.o. 5 x/day for 7–10 days	
Acyclovir	5 mg/kg intravenously every 8 hours for 5–7 days	Preferred route in normal host
Valacyclovir	400 mg p.o. t.i.d.	Reserved for severe cases
Famciclovir	1 gram p.o. b.i.d. x 7–10 days	
Recurrent episode		
Acyclovir	250 mg p.o. t.i.d. x 5–10 days	Limited clinical benefit
Valacyclovir	400 mg p.o. b.i.d. x 5 days	
Famciclovir	500 mg p.o. b.i.d. x 5 days	
Suppression	125–250 mg p.o. b.i.d. x 5 days	
Acyclovir	400 mg p.o. b.i.d.	Titrate dose as required
Valacyclovir	500 or 1000 mg p.o. 1 x/day	
Famciclovir	250 mg p.o. b.i.d.	
Mucocutaneous HSV in an immunocompromised patient		
Acyclovir	200–400 mg orally 5 x/day for 10 days	For minor lesions only
	5 mg/kg intravenously every 8 hours for 7–14 days	
Valacyclovir	400 mg p.o. 5 x/day for 7–14 days	
Famciclovir	500 mg b.i.d. p.o.	
	250 mg t.i.d p.o.	
HSV encephalitis		
Acyclovir	10–15 mg/kg intravenously every 8 hours for 14–21 days	
Neonatal HSV§		
Acyclovir	20 mg/kg intravenously every 8 hours for 14–21 days	

*The dose are for adults with normal renal function unless otherwise noted.
§Not currently approved by the FDA.
HSV, herpes simplex virus.

HSV infection from about 70% to 5–20% (275). A sequential regimen of intravenous acyclovir followed by oral acyclovir, valacyclovir, or famciclovir for 3–6 months can virtually eliminate symptomatic HSV infections in organ transplant recipients. A variety of oral dosing regimens (acyclovir 200 mg t.i.d. to 800 mg b.i.d.; valacyclovir 500 mg or 1 gram q.d. or b.i.d.; famciclovir 500 mg t.i.d.) have been used successfully. Among bone marrow transplant recipients and patients with poorly controlled HIV infection, acyclovir-resistant HSV isolates have been identified more frequently after therapeutic acyclovir administration than during prophylaxis.

Herpes Simplex Encephalitis

HSE is associated with substantial morbidity and mortality despite the use of antiviral therapy (144, 276). The administration of acyclovir at a dose of 10 mg/kg every 8 hours for 10–14 days reduced mortality at 3 months to 19%, compared with approximately 50% among patients treated with vidarabine (144). Furthermore, 38% of the patients treated with acyclovir regained normal function. Patients with a Glasgow coma score of less than 6, those over 30 years of age, and those with encephalitis longer than 4 days had a poor outcome. For the most favorable outcome, therapy must be instituted before semicoma or coma develops. Current recommendations utilize a 21-day regimen. Of note there is no benefit of long-term suppressive valacyclovir therapy (277), in contrast to that observed in neonatal infection.

Neonatal HSV Infections

Babies with CNS or disseminated disease when treated with 20 mg/kg every 8 hours for 21 days have mortality rates of 5% and 25% compared historically to 10% and 55%, respectively. Among those survivors, 35% and 80% of babies develop normally, respectively (278–280). No baby with disease localized to SEM died. Of note, PCR assessment of the CSF should be used to classify extent of disease (i.e., CNS involvement) as well as to identify SEM babies who have asymptomatic CNS involvement. Furthermore, assessment at the end of therapy should be performed on the CSF, because some newborns will require longer therapy. Chronic suppressive therapy (300 mg/m^2 t.i.d.) for 6 months has been proven effective in improving neurologic outcome as defined by higher Bayley Developmental Score (280).

Other HSV Infections

Case reports have described the successful use of acyclovir, valacyclovir, and famciclovir in the treatment of other HSV infections, such as hepatitis, pulmonary infections, herpetic esophagitis, proctitis, eczema herpeticum, erythema multiform, and herpetic whitlow (Table 1). Topical therapy with acyclovir for HSV ocular infections is effective, but probably not superior to trifluridine (281, 282) (Table 2).

Antiviral Resistance

HSV can develop resistance to acyclovir through mutations in the viral gene encoding TK, through the generation of TK-deficient mutants, or through the selection of mutants

TABLE 2 Investigation uses of acyclovir

Herpes labialis (prophylaxis)
HSV keratitis
Suppression of HSV infections in immunocompromised patients
Disseminated or visceral HSV infections (e.g., hepatitis)

HSV, herpes simplex virus.

possessing a TK that is unable to phosphorylate acyclovir (see Chapter 12) (283). Clinical isolates resistant to acyclovir are almost uniformly deficient in TK, although isolates with an altered DNA polymerase have been recovered from HSV-infected patients. Drug resistance was considered rare, and resistant isolates were thought to be less pathogenic until a series of acyclovir-resistant HSV isolates from patients with the AIDS were characterized (284). Although sensitive to vidarabine, cidofovir, and foscarnet *in vitro*, only foscarnet and cidofovir have been shown effective in the treatment of acyclovir resistant HSV. Acyclovir-resistant HSV isolates have been identified as the cause of pneumonia, encephalitis, esophagitis, and mucocutaneous infections in immunocompromised patients.

Toxicity

Acyclovir, valacyclovir, and famciclovir therapy is associated with very few adverse effects. Renal dysfunction has been reported, especially in patients given large doses of acyclovir by rapid intravenous infusion, but appears to be uncommon and is usually reversible. The risk of nephrotoxicity can be minimized by administering acyclovir by slow infusion and ensuring adequate hydration. Oral acyclovir therapy at doses of 800 mg five times daily and valacyclovir at doses of 2 grams b.i.d. have not been associated with renal dysfunction (285). A few reports have linked intravenous administration of acyclovir with disturbances of the CNS, including agitation, hallucinations, disorientation, tremors, and myoclonus (140).

The Acyclovir in Pregnancy Registry has gathered data on prenatal exposure to acyclovir. No increase in the risk to the mother or fetus has been documented, but the total number of monitored pregnancies is too small to detect any low-frequency events (286). Because acyclovir crosses the placenta and is concentrated in amniotic fluid, there is concern about the potential for fetal nephrotoxicity, although none has been observed.

New Therapies

There is a paucity of new drugs in development for the treatment of HSV infections. Only one, Pritelivir, a helicase-primase inhibitor, has been shown to have clinical and virologic value in a controlled clinical trial (287). However, because of concerns of toxicity in nonhuman primates, the FDA has put the drug on "clinical hold" (288).

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Cercopithecine Herpesvirus 1 (B Virus)

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B virus, endemic in macaque monkeys, has the unique distinction of being the only one of nearly 35 identified nonhuman primate herpesviruses that is highly pathogenic for humans. Infection has resulted in over 50 cases, with a mortality in excess of 80% in the absence of therapy. The actual number of cases worldwide is unknown. The unique biology of B virus includes its neurotrophism and neurovirulence. Because untreated B-virus infections are associated with high mortality in humans, individuals handling macaques or macaque cells and tissues are at risk for infection. Human infection is associated with a breach of skin or mucosa and subsequent virus infection. Fomites and contaminated particulates or surfaces can serve as a source of virus infection. Since the early 1980s, 80% of infected individuals given antiviral treatment have survived. Timely antiviral intervention is the only effective means of reducing B-virus-associated morbidity and preventing a fatal outcome.

HISTORY

In 1932, a young physician Dr. William Brebner was bitten by a monkey and later developed localized erythema at the site of the animal bite. This apparent localized infection was followed by lymphangitis, lymphadenitis, and, ultimately, a transverse myelitis with demise ascribed to respiratory failure. Autopsy tissue specimens from WB were obtained for laboratory investigation by Frederick P. Gay and Margaret Holden, who reported that an ultrafilterable agent recovered from neurologic tissues caused a cytopathic effect in tissue culture similar to herpes simplex virus (HSV) (1). The isolate was initially designated as "W" virus. Gay and Holden noted that it caused a similar disease in rabbits infected by either intradermal or intracranial routes of infection. Importantly, a rhesus macaque exposed to this virus showed no evidence of illness but developed antibodies, indicating asymptomatic infection.

Within a year of this first report, Albert B. Sabin independently also found an ultrafilterable agent in the tissue of this same index patient. He identified this as "B" virus (2), naming the virus by the initial of the patient's last name. Thus, the virus subsequently has been called Herpesvirus simiae, Cercopithecine herpesvirus 1, or more simply, B virus. Sabin also described the lethality of B-virus infection in rabbits, in which infectivity was independent of route of inoculation. Experimentally infected dogs, mice, and guinea pigs showed no susceptibility to infection, regardless of the route of inoculation (2). Both groups observed that B virus induced immunologic responses in an infected host similar to HSV (3). The virus was also noted to share similarity with pseudorabies, among other viruses, including SA8 and two additional more recently described nonhuman primate alphaherpesviruses, human papillomavirus 2 (HPV-2) and Langur herpesvirus (3–8).

By 1959, B virus had resulted in 12 cumulative fatalities with 5 recognized survivors (9–11), suggesting that this infection was not always fatal. Although B-virus antibodies were detected in a number of individuals who had no clinical symptoms but a history of working with macaques (12), the early serologic assays could not distinguish between HSV and B-virus infection. Once differentiation of humoral immune responses to these viruses was possible, a high-risk group of 325 subjects exposed to rhesus macques rarely, if ever, had antibodies in the absence of clinical symptoms.

During the decades since the initial reports of B-virus infection, investigators have defined the molecular biology of this virus and the resultant illness in humans and nonhuman primates, including its natural host, the macaque monkey. Because of neurovirulence in a foreign host, B virus is considered by some to be one of the most dangerous occupational hazards for those who work directly with macaques or their tissues or with monkeys who have been in contact with macaques.

VIROLOGY

Isolation and Growth Properties

According to the CDC guidance, isolation of the virus is recommended in a Biosafety Level 3 (BSL-3) laboratory, whereas propagation should be strictly confined to a BSL-4 facility (39). The initial isolation of B virus was achieved using rabbits for passage of the virus (1, 2, 40); shortly thereafter, the virus was also grown on chorioallantoic membranes of embryonated eggs (4).

In 1954, B virus was isolated from rhesus kidney tissue that was used for preparation of poliomyelitis vaccines (41) and from rhesus central nervous system (CNS) tissue (42). Subsequently, cultured cells derived from monkey kidney and chick embryo were found to support the replication of B

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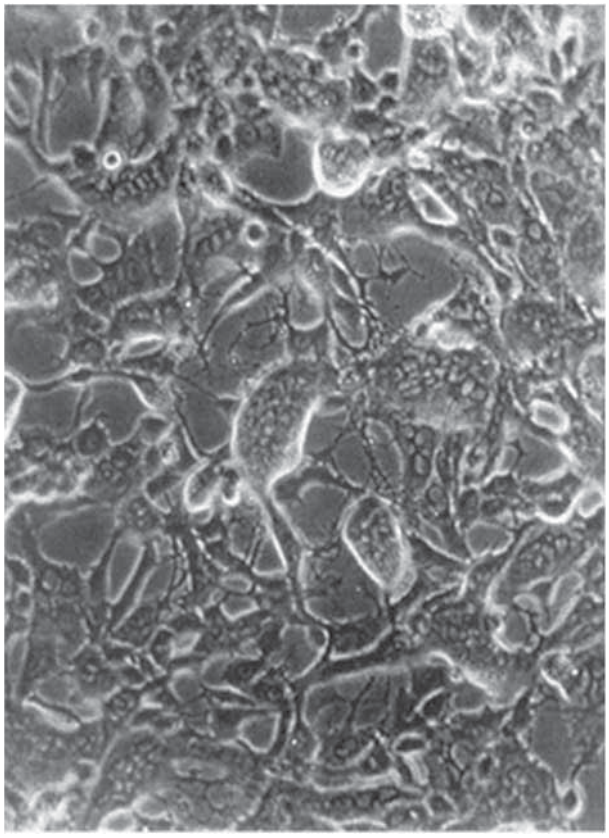

FIGURE 1 Vero cell monolayer infected with B virus.

virus (43, 44). The virus is stable in cell culture media stored at 4°C and can be maintained for years at −80°C but not at −20°C.

Replication kinetics of B virus follows a time course similar to HSV *in vitro*, with Vero cells being the best system for propagation (3, 4, 7, 8, 45, 46). Host cell machinery ceases once virus enters the cell in a fashion similar to HSV (47). Notably, glycoprotein D (gD) is functional, but dispensable, for virus entry into macaque and human cells, varying slightly from HSV (48). An eclipse of cell and viral activities is noted during the first 2 to 3 hours after infection, followed by synthesis of polypeptides early during infection. By 4 hours after infection, DNA synthesis increases dramatically and in parallel with the synthesis of viral polypeptides. Morphogenesis is also similar to HSV, as shown by studies using electron microscopy (49). Infectious virus is detectable within 6 to 10 hours after infection, and both extracellular and intracellular virus levels plateau about 24 to 28 hours after infection, declining thereafter (44, 50). B virus, like HSV, has been observed to express sequential classes of proteins, that is, immediate early, early, and late proteins (50), and to possess glycoproteins and other structural proteins that were studied in detail with respect to their antigenic relatedness to HSV and later to other nonhuman primate alphaherpesviruses (51–54).

B virus replicates to high titer in cell lines of Old World monkeys, particularly in Vero, African green monkey, and vervet kidney cell lines, as well as rabbit kidney, BSC-1, and LLC-RK cells (31, 55). Although B-virus replication in cultured cells usually results in syncytium formation (Fig. 1), some primary isolates produce only cell rounding. Generally, cells balloon, fusing into polykaryocytes that expand outwardly as more cells become infected. In this manner, virus infection spreads through the entire cell monolayer, destroying cells. Eosinophilic intranuclear inclusions (Cowdry type A) can be detected after fixation and staining infected cell monolayers; however, these inclusions are not observed either in infected animals or in some humans with zoonotic infection (56, 57). Thus, the presence of characteristic intranuclear inclusion bodies is not a reliable diagnostic marker of infection.

The B-Virus Genome

B virus contains a double-stranded DNA genome of about 162 kb. One strain of virus originating from a cynomolgus monkey has been mapped and subcloned (58, 59). The genome contains two unique regions—unique long (UL) and unique short (US)—flanked by a pair of inverted repeats, two of which are at the termini and two located internally, an arrangement that results in four sequence-orientated isomeric forms, as occurs with HSV. Figure 2 shows the comparative genomic organization of B virus and HSV-1, as established by using HSV-1 genes to identify and locate specific B-virus homologs (58). The overall size of the genome is slightly larger than HSV-1 (152 kb) and HSV-2 (155 kb). The guanosine and cytosine (G+C) content of the DNA has been calculated to be 75% on the basis of the buoyant density of viral DNA (60). The location of genes within the UL regions of HSV and B virus is co-linear (58). Homologs of HSV *US9* and *US10* genes are located upstream of the US glycoprotein gene cluster. This is in contrast to the downstream location of these genes in the HSV US region (58). Sequence analysis of the prototype strain (E2490), which originated from a rhesus macaque, however,

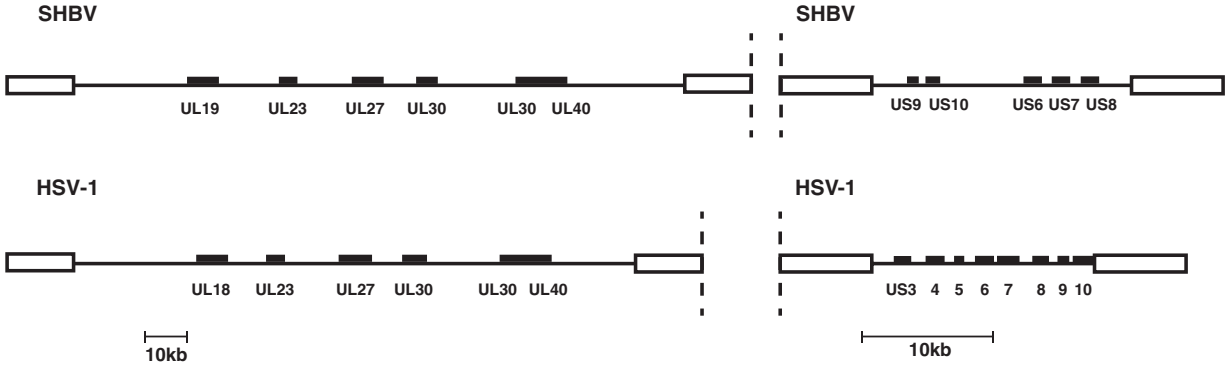

FIGURE 2 Physical map of B virus as proposed by Harrington and colleagues (58).

showed that B-virus DNA is colinear in these same regions with the HSV-1 genomic arrangement.

To date, sequences for only a few B-virus genes have been submitted to GenBank—homologs of gB, gD, gC, gG, gI, and gL—largely covering the sequence of the US region (61–63), although an entire genome sequence is available (59, 60). Each of the glycoproteins for which sequence information is available, except gG, has about 50% identity with HSV, slightly higher for HSV-2 than HSV-1. B-virus gG is a homolog of HSV-2 gG and is closer in size to gG-2 (699 kb) than gG-1 (238 kb) (63). Glycoprotein sequences demonstrate that all cysteines are conserved, as are most glycosylation sites. This conservation suggests that B-virus glycoproteins have similar secondary structure to that of HSV. Sequence analyses also suggest that B virus and HSV types 1 and 2 probably diverged from a common ancestor during the evolution of these pathogens.

Restriction-length polymorphisms (RFLPs) analysis indicates that intrastrain variation, commonly seen in HSV, exists among both human and nonhuman primate-derived isolates, although the significance of this remains to be defined (64–66). The existence of three distinct B-virus genotypes raises the possibility that these may vary with respect to pathogenicity for nonmacaque species (67). These genotypes were identified when collected data were analyzed phylogenetically; however, this postulate must be studied in a suitable animal model. Published case summaries that implicate other macaque species, and in one case a baboon, are difficult, if not impossible, to confirm (24).

Viral Proteins

More than 50 polypeptides ranging from about 10,000 to 250,000 daltons have been identified by immunoblot analysis of infected cells. Each has been assigned an infected cell polypeptide number as an initial reference point (50). This number may be an underestimate of the total synthesized, but it serves as a basis for comparison in ongoing studies. More than 75% of the expected coding capacity of the viral DNA is accounted for by these infected cell polypeptides. Many of these glycoproteins have been cloned and sequenced (61, 67, 68). The proteins encoded were mapped to genes in the US region, which was largely colinear with the HSV glycoproteins gD, gI, gJ, and gG, as previously described. Sequence analysis of selected genes showed that B virus is most closely related to herpesvirus papio 2 (HVP-2) (69). There are protein homologs in herpesviruses of New World monkeys. Very little, if any, cross-reactivity exists between B virus and the New World monkey herpesviruses.

The kinetics of protein and glycoprotein synthesis in infected cell cultures are similar to that observed for HSV, although infectious virus is detected earlier, appearing 6 hours after infection. Both host cell DNA and protein synthetic activities appeared curtailed during the first 4 hours after infection. The polypeptides of B-virus-infected cells, summarized in Table 1, may represent an overestimate of the primary gene products of the virus; however, this summary should serve as a fairly complete listing of B-virus-induced polypeptides for future reference. As for glycoproteins, only glucosamine and some mannoses are incorporated during the infection *in vitro*. B-virus polypeptides and glycoproteins can be grouped into classes that differ in their relative rates of synthesis at different times throughout the virus replication cycle, as is characteristic of alphaherpesviruses (50).

EPIDEMIOLOGY

Distribution

B virus is endemic in macaques, one of the Old World monkeys widely distributed throughout Asia. All species of macaques tested thus far serve as natural hosts. The virus can infect other nonhuman primates, but in such cases, the infected animal is not considered a natural host, and infection generally results in death within a relatively short time. Transmission of virus occurs by direct contact between members of the *Macaca* genus, from infected animal to human, from virus-contaminated surfaces, or, in one case, from human to human, as reviewed (13–26).

B-virus infection in the macaques rarely causes disease, and if so, it is mild ulcerative lesions. Infection in macaques is seldom associated with death, unless conditions prevail that facilitate generalized systemic infections (30).

Natural Hosts

Macaques

Most adult wild macaques are seropositive for B virus (28). Prevalence estimates of B-virus infection, using different serologic methods and sampling approaches in both wild and

TABLE 1 Hybridization of plasmids containing HSV-1 DNA fragments to simian herpes B virus DNA blot membranes

Probe DNA			SHBV fragment(s) to which probe hybridized	
HSV-1 KpnI clone	Fragment	Gene function	BamHI	SalI
h	SstI	Glycoprotein D (HSV US6)	g	i
h	SstI	Glycoprotein E (HSV US8)	g	p′n′
h	SstI	Glycoprotein I (HSV US7)	g	ip′
h	BamHI	Tegument phosphoprotein (HSV US9)	r	c′
h	BamHI	Virion protein (HSV US10)	r	c′
n	XhoI/AspI	Glycoprotein B (HSV US27)	d	pz
a′	NotI	DNA polymerase (HSV US30)	z	e′f
i	BstX	Major capsid protein (HSV US19)	j	s, y
s	KpnI/BglI	Ribonucleotide reductase 1 (HSV US39)	m	t
s	KspI/KpnI	Ribonucleotide reductase 2 (HSV US40)	p	t

HSV-1, herpes simplex virus 1; SHBV, simian herpes B virus.

captive macaque colonies, indicate wide variability (27). Spread of infection in the colony correlates with onset of sexual activity, which facilitates transmission of the virus among animals (28, 29). Crowding of animals during transportation also accelerates the spread of infection (28).

Colonies of macaques that have been found to be predominantly seronegative do exist in the wild. More recently, such colonies have been established, apart from original natural habitats, to meet escalating needs for seronegative animals by the scientific community. These efforts have proven difficult (102). The seroprevalence in wild macaques, in addition to the noted high infectivity (27) and low morbidity within captive colonies (4), has confirmed the macaque as the natural host.

Macaques belonging to captive breeding colonies also have a high seroprevalence, in fact, one that increases after the onset of puberty (27, 101). Increasing seroprevalence appears to be associated with sexual transmission within the colony (89, 101). Infants and juveniles have a very low incidence of infection, judged by low prevalence of specific B-virus antibodies (27). There is evidence of transplacental antibody transmission. Importantly, all age groups have been found to be virus positive, indicating that routes of transmission other than sexual activity exist (76).

No particular species of macaque appears to be excluded as a natural host for B-virus infection, although there are minimal or no data available from certain species. While detection of antibodies has been confirmed in most macaque species, there has been speculation that virus isolated from certain species is less neurovirulent or less neurotropic than virus shed by rhesus macaques (103).

Virus shedding during either primary or recurrent infections has been noted to occur. In general, macaques shed virus for a longer duration during primary infection and, for short periods, even hours with recurrent infections. Levels of virus, as measured from mucosal swabs, range from 10^2 to 10^3 plaque-forming units (PFU). Asymptomatic excretion of virus occurs in slightly more than 2% of antibody-positive animals from mucosal sites and the eye. These observations suggest that reactivation is only transient (27–2931–38).

Humans

B virus is an infection that humans rarely contract, but when they do, 80% of untreated cases result in death. B virus is usually acquired through zoonotic transmission from either a macaque or infected cells or tissues from the animal. In one case, human-to-human transmission was attributed to a shared tube of antiviral medication that was applied to treat the patient's bite wound. Later, the same patient autoinoculated an eye, supporting the assumption that B virus can be transmitted in a fashion similar to HSV. A fatal case resulted from exposure of ocular membranes to virus from a monkey in the process of being transported. This tragedy has refocused attention on an earlier report implicating this type of transmission in the epidemiologic analysis of zoonotic transmission of the virus (43).

Interestingly, current case analyses suggest that categorization of risk levels with regard to the severity of injury is not useful. The low incidence of B-virus infection in humans makes it difficult to reach firm conclusions, but analyses of cases occurring during the 1990s support the observation that immediate medical attention ameliorates, if not prevents, disease. Only minimal disruption of the protective skin layer or instillation directly to a mucosal membrane can result in initiation of infection. The level of virus required to initiate infection in humans remains unknown. Rhesus macaques have been most frequently implicated as the source of infectious virus in the known human cases, but alone, this is insufficient to conclude that rhesus-derived virus is uniquely important in the establishment of zoonotic infections. Other species of macaques, including baboons, have been linked to fatal zoonotic infections (24).

The incidence of zoonotic infections has been correlated retrospectively with periods of increased usage of macaques for biomedical research. Evaluation of past cases underscores that transmission of the virus is often associated with no more than a superficial scratch or puncture, suggesting that once virus gains entry into a host, the ability to initiate disease is perhaps dose independent, at least in some cases.

PATHOLOGY AND PATHOGENESIS

During the course of B-virus infection, several observations are common to the natural host, the experimentally infected host, and infected humans. First, pathogenetic outcome varies according to the infected host. The route of inoculation predicts differences in the time course of infection, development, and spread through the CNS and visceral organs (e.g., spleen, adrenal, kidney, and, in some cases, even heart). These routes of infection are unique to the mode of infection—natural, experimentally infected, and human zoonotic infections. For example, venereal transmission of the virus is common in macaque hosts, whereas an intranasal route of virus delivery defines the experimental rabbit model, or, in the case of human zoonotic exposure, accidental aerosolization of virus. However, these routes share one common feature: All require exposure of mucosal membranes to infectious virus.

The cells that come into contact with the virus initially are also important to the permissiveness of infection. For example, nasal mucosa is a less ideal site for virus replication than lung (10,70–73). Another important consideration in B-virus infection is the quantity of the virus introduced initially into a host. For example, a far greater quantity of virus is required to infect rabbits by aerosolization than by an intradermal route of inoculation, although practically, whether these routes and doses influence human or nonhuman primate infection is unconfirmed. Another common feature of natural, experimental, or zoonotic infection is that B virus can be found in the CNS shortly after the onset of acute infection. But the distribution of virus and its consequences differs widely in natural infection as compared with infection failure of susceptible foreign hosts.

The Natural Host

The macaque suffers little or no morbidity as a consequence of infection. Exceptions occur rarely and appear to involve specific accompanying factors, such as immunosuppression or stress (30, 74, 75). Infection is self-limiting (22, 23, 29,76–78). Virus replicates at the site of inoculation and induces localized erythema. Limited focal infection of liver and kidney may occur in some macaques (22, 23). Virus travels through the peripheral nerves from the site of inoculation to the representative sensory ganglia. Latent infection is established in the ganglia with intermittent reactivation of the virus throughout the life of the macaque (17, 27, 32, 36, 45, 79, 80). In rare cases, viremia has been observed (74, 81, 82). Virus has also been recovered from urine as well as multiple organs of animals. Reactivation from latency occurs in the natural host as judged by increasing antibody titers, recovery of virus by cocultivation of

sensory ganglia, and isolation of virus in the absence of detectable lesions (30, 35, 83).

During active B-virus replication, isolation of virus is readily accomplished from buccal, conjunctival, or genital mucosa sites (76, 84). The frequency of active infection among seropositive macaques is low, with relatively short time periods for successful virus recovery from mucosal sites (27, 29).

Mucosal ulcers, if present, extend down to the papillary layer of the dermis. Two distinct zones have been described, namely a central area of necrosis and a surrounding zone of ballooning degeneration. "Normal" epithelium exists around the lesion. An eosinophilic polymorphonuclear infiltration characterizes the histopathology of the lesion. Postmortem histopathologic examination of monkey tissues from animals euthanized at the time of active virus shedding reveals perivascular lymphocytic cuffing in sections of spinal cord. Examinations of latently infected but healthy animals failed to detect virus excretion routinely from peripheral sites, but virus can still be recovered from sensory ganglia (79).

Experimental Infections

Rabbits, mice, rats, guinea pigs, and chickens have been studied as B-virus models, as previously mentioned (3, 40, 45). Disease is not a uniform consequence following B-virus inoculation of mice and guinea pigs; however, several strains of B virus that have increased virulence for the mouse have been identified. One strain, identified as E2490, was avirulent for rats and chickens; nonetheless, antibody developed after infection (19). Cotton rats infected by intraperitoneal, subcutaneous, or intracerebral routes succumbed to infection with selected strains. Rats had typical hind leg paralysis secondary to transverse myelitis similar to symptoms in the rabbit. Serially passaged human B-virus strains are capable of infecting mice, hamsters, and white chicks (85, 86). With respect to experimental infections, the rabbit is the most useful small animal model because virus replicates to high titers, making it a particularly good model for testing antiviral agents.

Using the rabbit or the mouse model, viral dose is important, depending on the route of inoculation, for establishment of infection. Experimentally infected animals inoculated intradermally with low doses of virus developed only erythema that resolved within a few days and was not associated with further apparent symptoms. In contrast, animals receiving a larger dose developed a necrotic lesion that was generally followed by CNS invasion (87, 88). B virus subsequently appeared in the regional lymph nodes late after infection. These nodes drained the area of initial infection, and, with time, necrosis of the infected nodes occurred, as seen upon postmortem examination. In the CNS, focal lesions are evident in pons, medulla, and spinal cord. Although virus spread through peripheral nerves is most common, in rare cases a hematogenous route of spread in experimentally or inadvertently infected hosts occurs (8, 30,87–89).

The cervical spinal cord and medulla oblongata are the primary sites of postmortem virus recovery. With time after infection, virus is also found in olfactory regions of the brain that may be the consequence of movement of the virus centripetally through the nerves innervating nasal mucosa. Perivascular cuffing and glial infiltration are characteristic histopathologic findings upon examination of brain tissue. Hepatic congestion is accompanied by infiltration of polymorphonuclear and mononuclear cells seen in the periportal areas of the liver. Scattered foci of necrosis can be found throughout the lobes of the liver. The presence of inclusions is detected mainly in the regions of inflammation around pyknotic or karyorrhetic hepatocytes. When lesions are present on skin, the depth of the involved tissue is significantly greater than that of mucous membranes, perhaps explaining the reason B virus can be recovered weeks or months later from these sites (56, 57, 90).

Human Infection

Human B-virus infection generally occurs through occupational exposure to a macaque shedding virus at a site that comes into contact with broken skin or mucosal membranes of the susceptible human. Several human cases in which monkey contact had not occurred in years suggest that virus can infect and subsequently be reactivated.

The most striking characteristic of human B-virus infection is the involvement of the CNS, specifically the upper spinal cord and brainstem. These areas are the principal sites for virus replication. Initially, the infected individual experiences an influenza-like syndrome followed by numbness or paresthesia around the site of inoculation. An ascending transverse myelitis occurs during the final stages of the infection in humans, resulting ultimately in respiratory failure. Virus can be recovered at skin sites of inoculation for extended periods of time, and viral DNA can be detected generally in cerebrospinal fluid (CSF) by the time neurologic symptoms are experienced; CSF antibodies can also be detected. Cutaneous vesicular lesions are a source of virus isolation, even late in infection (91). Edema and degeneration of motor neurons are prominent. Even with advanced disease, Cowdry type A eosinophilic intranuclear inclusions can be found in only a few cases and certainly not uniformly. Gliosis and astrocytosis are late histopathologic findings; thus, there can be evidence of myelitis, encephalomyelitis, or encephalitis, or combinations of each of these conditions (24).

In several reports, ocular disease has been reported (95, 96). Histopathologic examination of the eye revealed a multifocal necrotizing retinitis associated with a vitritis, optic neuritis, and prominent panuveitis. Herpes-like viral particles were identified in the involved retina by electron microscopy in one case. Postmortem vitreous cultures taken from both eyes and retinal cultures taken from the right eye in the later case were positive for B virus. Thus, B virus can infect and destroy retinal tissue similarly to other herpesviruses. Ophthalmic zoster-like symptoms have been reported as well (33), and in one case, reactivation of latent infection was postulated.

To summarize human pathogenesis, the tissues and organs that become infected by B virus vary, likely according to the route of inoculation. If skin is the primary site of infection, the virus usually, but not always, replicates in the skin, resulting in localized erythema. Knowledge of the site of initial replication is useful for the development of guidelines for disease prevention and also for retrieval of a virus isolate that then allows unequivocal diagnosis. Subsequently, lymphangitis and lymph node involvement are observed. Although viremia has been documented in rabbits and monkeys, it has not been proven in humans, although with the application of more sensitive assays (e.g., polymerase chain reaction [PCR]), more details of infection can be uncovered. Certainly, with lymphatic involvement, virus can spread, particularly to abdominal viscera, where it has been isolated. Nevertheless, spread through neuronal routes is the fundamental route of transmission of the virus, as it is with HSV, given the involvement of the spinal cord and

CNS. Visceral organs, including heart, liver, spleen, lungs, kidneys, and adrenals, demonstrate congestion and focal necrosis with variations in the extent of involvement from patient to patient. Recent human cases failed to demonstrate necrosis, but virus was isolated from adrenal, kidney, lung, and liver tissue collected at autopsy (56, 57). In cases in which B-virus infection is suspected, medical personnel should follow published guidelines at the time of injury or observation of symptoms of possible infection (97–100).

Immune Responses

B-virus antibodies have been studied in both natural and foreign hosts by a variety of methods, including serum neutralization with or without complement, competitive radioimmunoassay (RIA), multiple types of enzyme-linked immunosorbent assay (ELISA) including competition ELISA, and Western blot. Relatively consistent antibody responses are induced in both hosts (104–109). The natural history of antibody development has been measured in wild macaques, captive colony populations, individually imported animals, experimentally infected macaques, zoonotically infected humans, and even vaccine trial recipients. The ELISA methodologies provide a rapid diagnostic tool with increased sensitivities of detection and with enhanced specificity when competition protocols are used. Host humoral response to B-virus infections both in humans and nonhuman primates neutralize HSV-1 and HSV-2 as well as nonhuman primate alphaherpesviruses. Interestingly, HSV antibodies do not neutralize B virus, indicating the presence of virus-specific antigens unique to B virus (110, 111). Sequence data have been useful in confirming the existence of B-virus-specific epitopes (61–63).

The humoral immune response to B-virus infection has a characteristic pattern (56, 90, 112). The glycoproteins induce antibodies early in the course of infection. Antibodies begin to appear within 7 to 10 days after the infection and consist of immunoglobulin M (IgM). Within 14 to 21 days after the onset of acute infection, IgG antibodies are present. In rare cases, the infected host remains persistently antibody negative despite virus isolation. The pattern of the immune response is somewhat altered in the cases of humans who have had a previous infection with HSV-1 or HSV-2, because viral antigens that are shared among the three viruses induce an anamnestic response toward shared protein or glycoprotein sequences. Neutralizing antibodies develop in both the natural and foreign hosts, but at significantly lower levels in the foreign host. The nature and specificity of the humoral responses make it possible to design enhanced serologic testing strategies to identify detectable antibodies rapidly and to provide the basis for future diagnostic strategies.

Latency

A characteristic of all herpesviruses is the ability to establish latency and to reactivate when provoked by the proper stimulus. B virus is no exception. Reactivation has been described in both wild macaques and established captive colonies (25, 27, 29, 35, 101). Unequivocal evidence of latent B-virus infection in macaques came with studies on frequency of recovery of virus in monkey kidney cell culture systems. At least 1% of macaque kidneys harbor latent virus that can be reactivated in cell culture (41). Virus has been isolated from rhesus tissues (32, 79) as well as by cocultivation from a variety of neuronal tissues, including gasserian ganglia, trigeminal ganglia, dorsal route ganglia, and spinal cord (38). Latent virus has been isolated by cocultivation of tissues from experimentally infected rabbits (38), further supporting the rabbit as a potentially acceptable animal model for B-virus infections. Latency likely occurs after human infection.

As with human HSV infections, a prominent factor associated with reactivation of B virus in macaque monkeys appears to be stress, particularly that associated with the capture and shipment of animals from the wild to captivity. Shedding of virus after reactivation also occurs with illness and during the breeding season of the natural host. No information is yet available on the state of the viral DNA during latency or on the molecular or biochemical events associated with the establishment and reactivation of latent virus.

CLINICAL MANIFESTATIONS

Observations of the clinical pattern of disease are important for rapid diagnosis of B-virus infection in both macaques and humans.

Humans exposed to B virus demonstrate clinically variable signs of disease. Surviving cases have varying degrees of morbidity, ranging from little or no neurologic impairment to more extensive CNS involvement (9,92–94). Some survivors experience slow neurologic decline, whereas others report few if any long-term effects. Several infected individuals have subsequently given birth to healthy children with no ill effects in either mother or infant. Monitoring of the vaginal canal for virus shedding in these individuals before delivery has produced negative results. Most often, illness is apparent within days to weeks, but in some cases, there appears to be a delay in development of acute disease. The reasons for this delay are unknown, and, although rare, delays may even range from months to years, making diagnosis difficult. Once symptoms appear, the clinical progression is associated with relatively consistent symptoms, including influenza-like illness, lymphadenitis, fever, headache, vomiting, myalgia, cramping, meningeal irritation, stiff neck, limb paresthesias, and urinary retention with an ascending paralysis culminating in respiratory failure requiring ventilatory support. Cranial nerve signs, such as nystagmus and diplopia, are common in most published cases. Sinusitis and conjunctivitis have been observed in some (56, 90). The array of symptoms may be related to the dose of virus with which the individual was infected or the route of inoculation. A summary of descriptions of human cases can be found in two comprehensive reviews (24, 25).

The highest percentage of deaths occurs within a few weeks after onset of disease. In some cases, however, life has been prolonged artificially for months or years. Incubation times from identifiable exposures to onset of clinical symptoms ranges from days to years, but most cases occur within days to months. Virus has been recovered from throat, buccal, and conjunctival sites as well as from lesions, vesicles, or injury sites as late as weeks to months after infection. Most clinical cases are associated with bites (50%), fomites, (8%) saliva (< 5%), and aerosols (10%).

DIAGNOSIS

Nonhuman Primates

Macaques

B-virus infection in macaque monkeys is identified by virus isolation, the presence of specific antibodies, or both (65, 113, 114). The neutralization antibody test was the

predominant diagnostic tool in macaques and humans for many decades, but the time required for obtaining results was a drawback. Thus, dot blot, RIA, ELISA, and Western blots were developed (115–121); the results of these tests can be acquired in less than 1 day. Three of these techniques (dot blot, ELISA, and Western blot) rely on the use of monoclonal antibodies. These tests are available through commercial laboratories as well as through a national resource laboratory subsidized through the NIH National Center for Research Resources. All of these assays use B-virus-infected cells for antibody detection, making such tests more effective than other types of assays that rely on HSV-1 (122). This is a particularly important point with respect to diagnostic tools used to identify signs of infection for the establishment of B-virus-free colonies. Tests dependent on monoclonal antibodies or recombinant reagents have defined sensitivity and specificity for each macaque species to be tested. Finally, evaluation of a macaque is only optimal when analysis is performed on multiple samples at different dates, especially in cases in which antibody titer is low (less than 1:50). A constellation of different tests for deployment at varying time points after infection may be necessary in some cases to determine the status of an animal with a very low antibody titer correctly, particularly when such an animal is housed in a B-virus-free colony (123).

While virus isolation is the gold standard for diagnosis of infected macaques, it is not a particularly sensitive diagnostic tool and has the possibility of many false-negative results. Nonetheless, standard cell culture for virus isolation remains a valuable tool for the colony manager and for the veterinarian. Virus-positive cultures can be easily recognized by their unique cytopathic effect, but unequivocal confirmation requires either electrophoretic analysis of infected cell polypeptides or restriction-endonuclease-digested DNA. Several PCR tests have been described that can be used to verify the identity of the virus; however, this diagnostic tool for colony management is costly. Nonetheless, when a possible zoonotic infection must be confirmed, PCR may be beneficial for identification of B virus in macaques.

Nonmacaques

Other species of monkeys infrequently become infected with B virus. These are usually animals that have been cohoused or housed in close proximity to B-virus-infected macaques at some time. Because many, if not all, nonhuman primates harbor indigenous alphaherpesviruses, the important diagnostic point is to differentiate specific antibodies from cross-reactive ones. Euthanasia is generally advised in the case of a B-virus infection in a nonmacaque monkey because it is likely that the animal will succumb, and, in the meantime, would pose a great risk to anyone attempting to treat the infection. B virus has been identified in the patas monkey and colobus monkey (124–126). In each case, there is a major concern for the people responsible for care of the animal, particularly because these animals often have severe morbidity and are shedding virus. Currently, the most effective assay for diagnosis of B virus in a nonmacaque monkey is a competition ELISA to facilitate discrimination between specific and cross-reactive antibodies similar to the challenge faced when diagnosing infection in humans.

Humans

Both serologic and virologic techniques are available for diagnosis of B-virus infection in humans. Clinical symptoms in association with detection of specific antibody or virus positivity are the gold standard for diagnosis of B-virus infection in an exposed individual. The CDC has published specific guidelines for recognition and treatment of such infections (57). In the case of a suspected infection, several emergency resources are available. Contact with the CDC or laboratories recommended by the CDC (see CDC guidelines) can expedite laboratory assistance for the clinician suspecting B-virus infection.

Generally, a rise in B-virus-specific antibodies over several days during acute infection substantiates B virus as the etiologic agent. However, in other cases, data are equivocal, and decisions with regard to patient management must be based on a complex decision table collectively using all diagnostic tools, including clinical symptoms. Serologic diagnosis of B virus in humans is a complex task when an individual with a suspect infection has detectable antibodies as a result of a previous HSV infection (127). As discussed previously, significant cross-reactivity of host response exists among these viruses. In the absence of these cross-reactive antibodies, diagnosis is rapid and straightforward, with confirmation using the neutralization assay, Western blot, or both. This was not the case before the development of rapid diagnostic competitive ELISAs and RIAs (119, 120). The diagnostic tests for humans are performed currently by only a few facilities that have been licensed and have access to BL-4 containment laboratories for the preparation of B-virus antigen.

Virus isolation remains the gold standard for diagnosis but is frequently not possible, even under the best of circumstances. Virus identification can be accomplished by isolation using conventional cell culture and, in clinical emergencies, by PCR (128). The identity of isolates should be confirmed by electrophoretic analysis of infected cell polypeptides or restriction-endonuclease-digested DNA (50). The application of PCR is most helpful in the symptomatic patient if virus cannot be recovered. PCR is also a useful tool for monitoring the efficacy of antiviral interventions.

PREVENTION

Guidelines for treatment and prevention of B-virus infection can be accessed rapidly either through the CDC or the diagnostic resource using the URL www.gsu.edu/bvirus. Because of the risk of human disease, precautionary methods must be followed in the workplace. Proper attention to the details of housing, management, and handling of macaque monkeys and organized exposure response measures using the CDC guidelines can minimize B-virus zoonotic infections.

Exposure Risk Reduction

Multiple levels of prevention are used to prevent B-virus infection in both humans and nonhuman primates, ranging from attempts to eliminate virus-positive animals from colonies to designing methods to work safely in environments where there is increased risk for contracting this agent. Recognition of early infection allows removal of infected monkeys from captive colonies that are being established as B virus free. In B-virus-free colonies, it is important to remove seropositive animals and isolate animals with equivocal results to prevent infection of other colony members, or in seropositive colonies to minimize risk to humans who handle them. Macaques are not treated with antivirals because the high prevalence of infection makes it cost prohibitive.

In cases in which B-virus infection is suspected, medical personnel should follow published guidelines at the time of injury or observation of symptoms of possible infection

(97–100). The CDC has published detailed guidelines for maximizing protection of individuals working with macaque monkeys (97, 99). Furthermore, the NIH National Center for Research Resources has funded the development of B-virus-free colonies for NIH-funded research involving these animals in an attempt to eliminate this virus from colonies used for biomedical research. Nonetheless, B-virus-infected monkeys are plentiful and require handling that can be done safely if strict guidelines are followed, including barrier precautions.

When B virus is detected, decontamination can be accomplished with either heat or formaldehyde (11). Other virus inactivators include detergents and bleach, but individuals who are working in a decontaminated area should still be alert for injury prevention. Minimizing fomites decreases worker risk and reduces virus spread among animals. One B-virus infection in a human was acquired from a cage after the person sustained a scratch (57), underscoring that surface decontamination can play an important role in infection control.

Vaccines

As early as the 1930s, attempts were made to identify an effective vaccine for protection of individuals who could be exposed to this virus while working with macaques or their cells or tissues. Limited vaccine trials have been performed in volunteers (108, 109), and, although short-term antibodies were induced, they waned quickly. Thus, the vaccine was not pursued further. Recently, a recombinant vaccine was tested and found to induce antibodies in macaques, but the duration of antibody persistence and efficacy remain to be assessed (68).

Exposure Management

With respect to prevention, the value of first aid after a potential exposure due to a bite, scratch, splash, or other suspicious injury is very important. Guidelines for wound cleaning are described in detail by the CDC. Every institution working with macaques should have an injury protocol with immediate availability of first aid, a secondary care plan, and last, but not least, an infectious disease specialist who is a member of the institution's prevention and care response team.

Chemoprophylaxis

Antiviral therapy is recognized as an effective prevention prophylactic of infection in human and animal trials when administered early after exposure (56, 57,129–131). Acyclovir and the related family of nucleoside analogs were noted to be effective when given in high doses (90), for example, acyclovir at 10 mg/kg intravenously three times daily for 14 to 21 days. Antivirals are used by an increasing number of facilities for postinjury prophylaxis or after laboratory results indicate an animal may have been actively infected around the time of the exposure. Postinjury prophylaxis has been performed with famciclovir or valaciclovir, and both have demonstrated efficacy *in vitro*.

TREATMENT

Antiviral therapy is uniformly used in humans with clinical disease. Efficacy of therapy in cases of infection in humans has been monitored by inhibition of peripheral virus shedding in some cases and by reduction in cerebrospinal fluid antibodies or viral DNA load in others (56, 57, 132). Some physicians follow intravenous therapy with long-term oral suppression (acyclovir, valaciclovir, or famciclovir). Ganciclovir has a greater efficacy *in vitro* and thus has been used in a few cases since 1989 with success. Interestingly, before 1987, in at least five retrospectively analyzed cases, individuals fared well in the absence of antiviral therapy, but the use of one of these nucleoside analogs is recommended by CDC.

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Varicella-Zoster Virus

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Varicella-zoster virus (VZV) is the etiologic agent of two diseases, varicella (chickenpox) and zoster (shingles). Varicella, which occurs after the initial encounter with VZV, is a disease manifested by a pruritic rash accompanied by fever and other systemic signs and symptoms that are usually mild to moderate in nature. Most often, but not always, varicella is a self-limited infection of childhood. Live attenuated varicella vaccine was licensed for routine use in the United States in 1995 and after more than 20 years of use has changed the epidemiology of the disease, as the incidences of varicella and its complications have now significantly declined (1–3).

Zoster is mainly a disease of adults. A prerequisite for developing zoster is a prior episode of varicella, which on occasion may have been subclinical or following varicella vaccination. During primary infection, VZV establishes a latent infection in sensory, cranial, and enteric neurons; this infection may result from invasion of nerves by VZV in skin vesicles or from a viremia with VZV (or both) (3). During latency the neurons do not show obvious effects from the virus. Zoster results when the latent virus reactivates into an infectious form and travels down the nerve (retrograde transport) to infect the skin. Most often VZV reactivates when the host is relatively immunologically compromised, particularly in cell-mediated immunity (CMI), as occurs with aging and following disease or various treatments such as steroids, cancer chemotherapy, transplantation, and irradiation (3, 4).

Varicella was distinguished clinically from smallpox in the mid-18th century. The origin of the name chickenpox is uncertain, but it may have been derived from the French pois chiche (chick pea), or from the domestic fowl (in Old English cicen and Middle High German kuchen). Herpes is derived from the Greek word meaning to creep; zoster comes from the Greek word for belt, and the word shingles is derived from the Latin word (cingulus) for girdle (3).

The delineation of the link between varicella and zoster is of virologic, medical, and historical interest. In 1888 Bokay recognized that cases of varicella often occurred following an exposure to patients with zoster and postulated that there was a relationship between the two diseases (3). In early attempts to develop a vaccine against varicella, medical investigators inoculated vesicular fluid from zoster patients into varicella-susceptible children, who subsequently developed chickenpox (3). Weller and Stoddard performed the first successful *in vitro* studies and showed that viruses isolated from patients with varicella and zoster are immunologically similar (5). In the mid-1940s a possible analogy with herpes simplex virus (HSV) infection was recognized, and it was proposed that zoster was resulted from reactivation of latent VZV (6). Hope-Simpson was the first to recognize the importance of the immune system in controlling manifestations of zoster; he postulated that zoster resulted from waning immunity to VZV in the years following varicella, permitting the latent virus to emerge (7). More recently the importance of declining cellular immunity to VZV was recognized in the pathogenesis of zoster (3). Using molecular techniques for DNA analysis, it was demonstrated that latent VZV is present in sensory neurons of individuals with a history of varicella (8). VZV DNA from zoster isolates was shown to be similar to the DNA of the virus that caused the primary VZV infection or the DNA of the virus used for vaccination, proving that zoster results from reactivation of latent VZV (3). It is widely accepted that zoster is not acquired from contact with patients with chickenpox. In a placebo-controlled study of vaccination to prevent zoster in the elderly with live attenuated VZV, the vaccinees were not predisposed to develop early zoster as compared to controls (4).

VIROLOGY

Classification

There is only one serotype of VZV; no differences in antigenicity among virus isolates have been identified. There are minor differences in DNA sequence among VZV isolates, and seven genotypes have now been identified, which on the basis of single nucleotide polymorphisms are classified as European, Japanese, or Mosaics (9). Analysis of 130 circulating VZV strains from the United States in 2001 and 2002 indicated that 81.5%, 3%, and 15.5% were of the European, Japanese, and Mosaics genotypes, respectively (10). The Oka vaccine strain has not been demonstrated to circulate.

The genomes of the parental (wild-type) and vaccine Oka strains have been fully sequenced; there are numerous differences between the parent and vaccine strains, especially within open reading frame (ORF) 62 (3). Fifteen of 42

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(36%) base differences between vaccine and wild-type VZV were present in ORF 62 (11).

The wild-type VZV DNA sequence is readily distinguished from that of vaccine virus by restriction enzyme analysis of DNA from cultured virus and/or by PCR. In countries other than Japan, a useful marker for the vaccine strain is the absence of a PstI restriction site in ORF 38 (3); however, this assay may become less useful should there be significant circulation of Japanese wild-type VZV in the United States. The presence of SmaI and NaeI restriction sites in ORF 62 clearly discriminates between the Oka vaccine strain and all wild-type strains, including Japanese wild-type virus (3). Using DNA sequence analysis of a single nucleotide polymorphism at 106262 in ORF 62, it is possible to distinguish rapidly between vaccine and wild-type VZVs (12). Molecular assays are useful for identification of adverse events temporally associated with varicella vaccine (3, 13) (3)

Composition of the Virus

Enveloped virions are 150 to 200 nm in diameter, with a central DNA core. The inner viral nucleocapsid has an approximate diameter of 100 nm, consisting of 162 hexagonal capsomeres with central axial hollows organized as an icosahedron with 5:3:2 axial symmetry (3). Consistent with the morphological structure of other herpesvirus virions, a biologically important coat, the tegument, surrounds the nucleocapsid, which is in turn surrounded by an envelope derived in part from cellular membranes.

The genome of VZV is composed of approximately 125,000 bp. It contains 74 ORFs, which account for 71 different gene products (3). The linear double-stranded DNA consists of a long unique segment of approximately 100 kilobase pairs (kbp) and a short unique sequence of approximately 5.4 kbp, flanked by internal and terminal repeats of 6.8 kbp (Color Plate 30) (14). It exists in four isomers; the predominant ones account for 95% of the VZV population (3).

By analogy with HSV, VZV is thought to replicate via a temporally regulated cascade of gene expression, consisting in general of synthesis of immediate-early (IE), early (E), and late (L) genes. Expression of L genes culminates in lytic, productive viral infection. Interruption of the cascade, particularly at the IE/E stages, can result in failure to synthesize infectious virus and may result in latent infection (15). IE genes are for the most part regulatory, E genes may be regulatory or structural, and L genes encode structural elements of the virion. Some of the IE regulatory gene products that also encode structural elements of the virion, such as the regulatory ORFs 62, 4, and 63, are also found in purified virions (16) (Table 1). Gene products of ORFs 21 and 29 are lytic-cycle genes expressed early in infection. The ORF 62 protein acts as a transcriptional activator or as a repressor and is required for further latent or lytic viral synthesis because IE gene 62 is thought to initiate the cascade of gene expression of VZV (15). Gene 61 is a transcriptional repressor (15) that encodes regulatory proteins; however, unlike the ORF 62 protein, the ORF 61 protein is not present in the virion (16).

The functions and biochemical characteristics of most VZV proteins are still unknown. Many are nonstructural proteins involved in different aspects of viral replication. About 30 polypeptides have been detected in VZV, at least 9 of which are glycosylated (17). The virus-encoded glycoproteins are named alphabetically, corresponding to those of HSV, as follows: gB, gC, gE, gH, gI, gK, gL, gM, and gN. There is no VZV equivalent of gD, importantly, which is the major glycoprotein of HSV. Some of these are essential to viral synthesis (such as gE) and others less so (such as gC). Some glycoproteins contain amino acid signal sequences or acid-rich patches in their cytoplasmic tails that are trafficking motifs that direct their movement, required for envelopment, to structures such as the *trans*-Golgi network and the plasma membrane (18–21).

TABLE 1 Summary of genetic information on VZV

Protein	ORF(s)	Comments on likely importance
gB	31	Fusion, entry, egress
gC	14	Adhesion, cell entry
gE	68	Most abundant; essential for replication, envelopment, spread
gH	37	Fusion, entry, egress
gI	67	Travels with gE; needed for proper envelopment
gK	5	Essential
gL	60	Travels with gH
gM	50	
Nucleocapsid	20, 40	
	10	Tegument, transactivator
	61	Transcriptional repressor
	28	DNA polymerase
Latency associated		
IE		
	63	Major protein; down-regulates transcription of IE gene 62; tegument
	4	Tegument, transcriptional activator
	62	Tegument, transcriptional activator or repressor
E		
	21	Nucleocapsid protein
	29	DNA binding protein
	66	Protein kinase

The most abundant glycoprotein of VZV is gE; encoded by ORF 68, it is essential for production of infectious virus (22). This glycoprotein exists as several glycopeptides in different maturational stages with molecular masses ranging from 60 to 98 kDa (23). It is highly immunogenic; it is a phosphorylated high-mannose O-linked and N-linked complex-type glycan which, in concert with gI, binds to the Fc fragment of human immunoglobulin G (IgG) (23, 24). Glycoproteins gE and gI are covalently linked and act as an Fc receptor on infected cells. They play an important role in cell-to-cell spread of virus and are also essential for envelopment (19, 25). They play roles in viral maturation, transportation of other glycoproteins to the cell surface, and virulence (19, 22, 26). Glycoprotein gE contains trafficking sequences that mediate assembly of viral proteins and envelopment in the *trans*-Golgi network (20, 21). It also (along with gI) acts as a navigator, directing additional glycoproteins to the cell surface and back to the *trans*-Golgi network, where final envelopment of virions occurs (19). A variant VZV with a mutated gE has been isolated from five individuals (27, 28). These viruses are thought to be escape mutants, and their biological significance needs further study.

The second most abundant glycoprotein is gB, a heterodimeric glycopeptide linked by disulfide bonds. It too plays important roles in viral entry, envelopment, and egress from cells (29). This glycoprotein is thought to have an important role in virus entry, egress, and cell-to-cell spread in VZV infection (30). It exists as a single-species glycopeptide with a molecular mass of 118 kDa (31); it is a high-mannose N-linked glycopeptide which does not contain O-linked oligosaccharides. Glycoprotein gI is a 60-kDa glycopeptide with both N-linked and O-linked oligosaccharides; it is nonessential for growth of VZV in fibroblasts, but mutants lacking it replicate poorly in cell culture (31). Glycoprotein gC is a heterogeneous 95- to 105-kDa glycopeptide that contains N-linked oligosaccharides and possibly O-linked oligosaccharides (32). Antibodies to gE, gI, gB, and gH have neutralizing activity and are therefore hypothesized to be critical for viral spread, particularly viral attachment and penetration (30–35).

Biology

Entry of cell-free VZV particles appears to be dependent upon several factors. These include cell surface heparan sulfate proteoglycan (36), calcium-independent mannose 6-phosphate receptors (MPR^{ci}) (36, 37), and the cellular insulin-degrading enzyme, which is a receptor for gE and is critical for cell-to-cell spread of VZV (38–40). Entry of cell-free enveloped virions into cells is highly cholesterol dependent and occurs mainly by clathrin-mediated endocytosis (Figure 1) (41).

After VZV DNA reaches the nucleus, VZV commences replication, synthesizing its DNA core and capsid. The capsid receives a temporary envelope from the inner lamella of the nuclear membrane; in order for it to receive a tegument it is de-enveloped by passing through the rough endoplasmic reticulum into the cytoplasm as a naked nucleocapsid (42). Nucleocapsids and glycoproteins are transferred independently to the *trans*-Golgi network, where the tegument is added and final envelopment occurs (Figure 2). Additionally, because of the presence of mannose 6-phosphate in VZV glycoproteins, there is an interaction between MPR^{ci} and VZV that results in the inclusion of virions into late endosomes, where they are exposed to lysosomal enzymes and an acid environment, and the infectivity of the virions thus becomes compromised (37). Most VZV synthesis occurs in this manner, *in vitro* and *in vivo*. In cell cultures, infectious VZV is not released into media. In contrast, in human skin, during the natural maturation of superficial epidermal cells, MPR^{ci}s are lost, along with the lysosomal pathway. In patients infected with VZV, free virions are produced in these cells and are released into skin vesicles during acute varicella

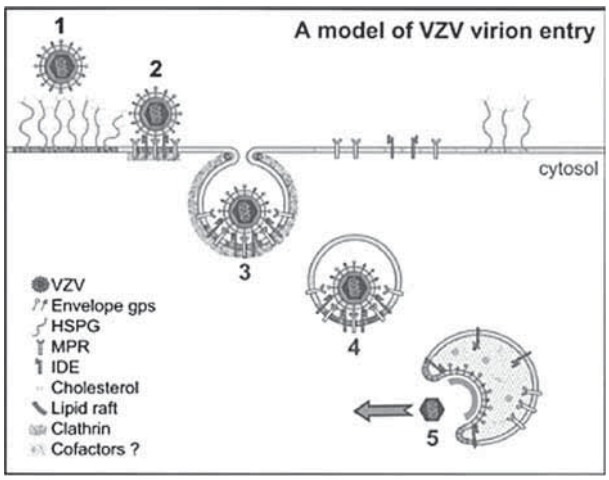

FIGURE 1 A suggested mechanism of VZV virion entry. (1) The incoming virion initially attaches to cellular heparan sulfate proteoglycan (HSPG), facilitating specific interaction with cellular receptor, e.g., MPR^{ci} (2). This leads to receptor-mediated (clathrin-dependent) endocytosis (3) and delivery to an endosomal compartment (4). Within this structure, the virion is exposed to cofactors for membrane fusion, possibly including the insulin-degrading enzyme (IDE) and/or altered pH. (5) Following triggered membrane fusion, the VZV nucleocapsid is delivered to the cytoplasm. Cholesterol may play a role at each stage. (Reprinted from reference 41 with permission.)

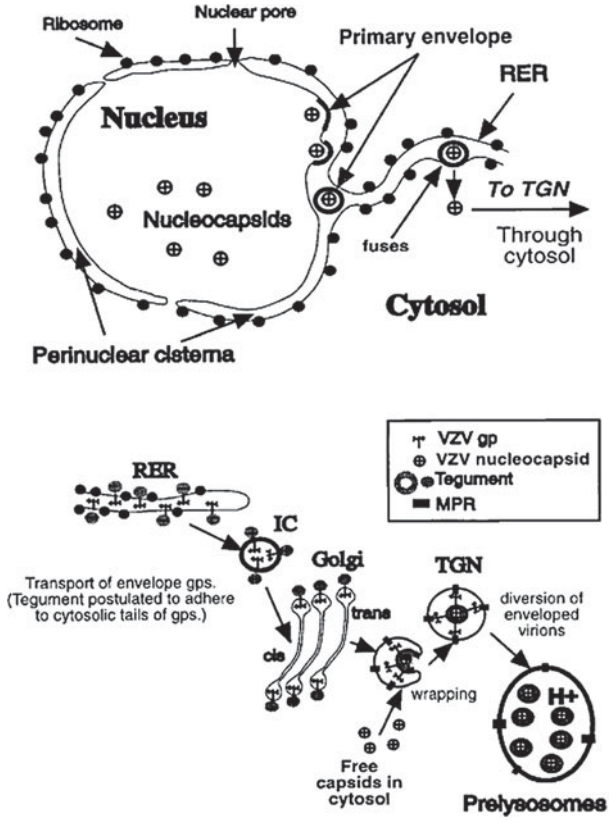

FIGURE 2 Putative route of intracellular transport and maturation of VZV in human embryonic lung fibroblasts. Structures are not drawn to scale. (Top) Nucleocapsids assemble in the nuclei of infected cells and bud through the nuclear envelope to reach the perinuclear cisterna-rough endoplasmic reticulum (RER). A temporary, primary viral envelope is acquired from the inner nuclear membrane. This envelope fuses with the RER, delivering unenveloped nucleocapsids to the cytosol. The nucleocapsids move through the cytosol to reach the *trans*-Golgi network (TGN). (Bottom) The glycoproteins (gps) of the viral envelope are synthesized in the RER independently of the nucleocapsids and are integral membrane proteins. Tegument proteins are probably synthesized by free ribosomes and are transported to the Golgi stack, as are the gps. Virions receive their final envelope by wrapping the TGN-derived sac and are delivered to acidic structures, identified as prelysosomes. (Reprinted from reference 152 with permission.)

and zoster. These 200-nm particles probably play roles in airborne transmission of VZV and establishment of latent infection in neurons (37, 43). If this hypothesis regarding MPR^{ci}s is correct, it may explain why infectious virions are not released into supernatant media in cell cultures and *in vivo* in cells where MPRs are abundant. In contrast, *in vivo* in the superficial epidermis, the lysosomal pathway is circumvented and infectious, cell-free VZV is released into human skin lesions, accounting for the high degree of infectivity of varicella and establishment of latent infection by infection of neurons, with axons present in the area of the epidermis where the cell-free virions are present (37).

VZV appears to spread, therefore, in two ways (43). It can spread from one infected cell to another cell by fusion of the two cells; fusion is mediated in all probability by gE, gI, gH, and gB on the membrane of the infected cell. This form of spread does not require envelopment of virions. In mutant VZVs lacking gI, cell-to-cell spread takes place, although proper viral envelopment does not occur (19). Cell-to-cell spread is thought to be the major means of spread of VZV in the body and would explain the importance of cellular immunity rather than antibodies in host defense against this virus. The other form of spread, described above in the discussion of viral entry, is by cell-free virus. It is hypothesized that synthesis of cell-free VZV occurs only in skin vesicles, because MPRs are not formed in the superficial epidermis, and therefore infectious enveloped virions are not exposed to the acid environment of endosomes, so they are released from infected epidermal cells in an infectious form (37). Cell-free VZV in skin vesicles can be aerosolized and thus spread to persons susceptible to varicella. Cell-free virions are also able to establish latent VZV infection in an in vitro neuronal animal model (43).

Latency of VZV differs from latency of HSV. While early information suggested that latency might occur in various cells, it is now widely accepted that latent VZV infection occurs exclusively in neurons (44–46). At least six VZV genes have been shown to be expressed during latent VZV infection in a number of studies; these are mostly IE genes (ORFs 4, 62, and 63). The IE ORF 61, however, is not expressed in latent infection. ORF 63 protein, the predominant latency protein, in some studies interestingly inhibits apoptosis of neurons infected with VZV *in vitro* (47). Some E genes are also expressed in latent infection (ORFs 21, 29, and 66), but L genes are not; their expression indicates lytic rather than latent infection (15, 43, 48–52). All of the VZV latency genes have HSV type 1 counterparts; however, none of these genes is expressed in HSV latency. In contrast to VZV, during HSV latency only one region of the genome, termed latency-associated transcript, is expressed (53). During VZV latency, it is hypothesized that the cascade of viral synthesis is interrupted and that there is a block in the synthetic cascade, perhaps at the level of expression of ORF 61 (15). VZV latency-associated proteins are present only in the cytoplasm. The mechanism responsible for this aberrant cell localization is poorly understood. At least one latency-associated protein, ORF 29 protein, however, can be made to enter the nuclei of latently infected neurons in response to inhibition of the proteasome or ectopic expression of ORF 61p (54). Dephosphorylation of ORF 62 protein causes this cytoplasmic protein to translocate to the nucleus (55).

While there are considerable data to indicate that during VZV latency there is some gene expression, exactly what is occurring is not fully understood. There are currently two general hypotheses concerning how latent infection is established. One is that neurons are infected by VZV and that a certain level of viral multiplication takes place prior to the virus becoming latent (56). The other posits that cell-free virions are transported to a neuron, and latency is established but multiplication does not occur. HSV is thought to establish latency without viral replication (57), although this model fails to account for the rather high number of genomes that are present in each latently infected cell. In an experimental *in vitro* model of VZV latency in guinea pig enteric neurons, following infection with cell-free VZV the virus does not replicate, latency results, and the neuron survives *in vitro* for weeks. Infection of neurons with cell-associated VZV, in contrast, results in replication with rapid neuronal death, despite the presence of ORF 63 protein, which becomes nuclear (43). Reactivation with production of infectious VZV and rapid neuronal death was demonstrated in an *in vitro* guinea pig neuronal model of latency, induced by infection with an adenovirus vector carrying VZV ORF 61 (43).

Whether VZV proteins are produced during latent infection has recently been questioned (58, 59); however, there seems to be agreement that RNA transcripts are produced (53). Several cytoplasmic VZV proteins have been demonstrated in the guinea pig neuronal model (43).) In patients undergoing gastrointestinal surgery, VZV transcripts of IE and E genes have been found in the enteric nervous system (ENS) in ~90% of those who either had a history of varicella or had been vaccinated, but they were not found in infants who had never been infected with VZV (60, 61). Further research on latency of VZV hopefully will clarify the participation of VZV transcripts and proteins during latent infection.

Other important observations with regard to VZV latency include the following. Patients with impaired CMI to VZV have an increased incidence of herpes zoster, which is consistent with the hypothesis that at least some aspects of suppression of VZV infectivity are under immunologic control (4). Various rodent animal models of VZV latency have been developed, but reactivation of the virus has not been demonstrated (3, 62). A recent exception is latent infection in guinea pigs, in which VZV has been shown to reactivate (63). See below.

VZV has an extremely limited host range, infecting mostly primates. Hairless guinea pigs may be infected with VZV that has been adapted in tissue culture to cells from this species. The primary illness produced is extremely mild and latent infection occurs only infrequently, but specific immune responses can be demonstrated (3). When guinea pigs are infected with VZV by intravenous injection of T lymphocytes infected with VZV, a latent infection occurs. Animals with latent infection appear asymptomatic. When they are treated with tacrolimus and cortisone-stimulating hormone, however, the latent VZV reactivates to cause a disseminated form of VZV infection in which the animals develop marked weight loss and rashes (63). It is hoped that in the future these animal models will provide information on VZV pathogenesis and that they may be used for testing various therapies and vaccines.

In vitro, VZV spreads rather slowly, from one cell to another. After inoculation of diploid human cell cultures such as human embryonic lung fibroblasts, a focal cytopathic effect (CPE) is not observed until at least 48 h after infection (Figure 3). VZV is best propagated at 32°C (3). The CPE of VZV in cell culture is characteristically focal, the result of cell-to-cell spread. Characteristic CPEs consist of foci of swollen refractile cells in a spindle-shaped configuration. Foci of infected cells enlarge and may slowly involve much

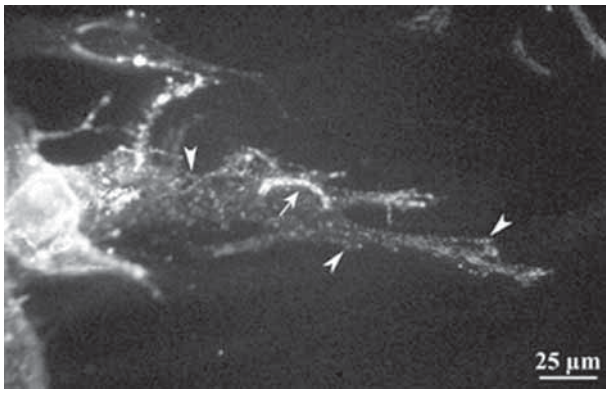

FIGURE 3 VZV propagated in human embryonic lung fibroblasts after several days in culture. The specimen was stained with fluorescein-tagged monoclonal antibodies to gE of VZV. The arrow indicates gE in the *trans*-Golgi network, and the arrowheads indicate gE at the cell surface.

of the monolayer, but infectious virions are not released from cells. Diploid human cell cultures are the most convenient host system for virus isolation; primary and heteroploid cell lines such as MeWo cells can also be used (64).

There is little available information on the stability of VZV, but it is usually classified as a rather labile agent. Virus obtained from vesicular fluid is stable for years when frozen in sterile fat-free milk at—70°C. Cell-free VZV obtained by sonication of infected cells can be stored at—70°C in tissue culture medium containing fetal bovine serum with sorbitol at a final concentration of 10%. Infected cells can be preserved at—70°C or in liquid nitrogen in medium containing 10% fetal bovine serum and 10% dimethyl sulfoxide.

EPIDEMIOLOGY

Distribution, Geography, and Seasonality

VZV infections occur worldwide. The virus spreads less readily in countries with tropical climates than in those with temperate climates (3), resulting in a higher rate of susceptibility to varicella in adults in tropical countries than in countries with cooler climates. It is possible that spread of VZV is inhibited at high temperatures because the virus is labile. In the prevaccine era, about 4 million cases of chickenpox—an entire annual birth cohort—were estimated to occur yearly in the United States. The disease affects males and females equally. Varicella is most common in the winter and early spring. In contrast, zoster occurs at equal rates during all seasons of the year (3).

Incidence and Prevalence of Varicella

Varicella is one of the most contagious of infectious diseases (65). Reported secondary attack rates of varicella following household exposure of susceptible contacts range from 61% to 100% (3, 65). Secondary varicella cases in a family are usually more severe than primary cases (65), presumably because the intensity of exposure in that setting results in a higher viral inoculum. The first child developing varicella in a family thus usually has a milder infection than subsequent family members who become ill (3).

About three fourths of U.S. adults with no history of varicella have detectable antibodies to VZV (66), indicating that subclinical varicella can occur. From an epidemiological study in which parents with no history of varicella were exposed to their children with chickenpox and did not contract it themselves, it was estimated that the incidence of subclinical varicella is approximately 5% (65).

In countries with temperate climates where vaccine is not used universally, varicella is a disease of childhood; most cases occur in children before they are 10 years old, with 8% to 9% of children annually developing disease between the ages of 1 and 9 years (3). These data were representative of children in the United States before many began to attend daycare facilities; it is possible that exposures to VZV in the daycare setting lead to an earlier acquisition of disease. The effect of daycare, however, on the epidemiology of varicella may never be fully known because live attenuated varicella vaccine was licensed for use in all varicella-susceptible children in 1995 in the United States.

Second attacks of varicella are uncommon but have been reported (67). Second attacks may be more frequent in immunocompromised hosts than in healthy individuals. One hypothesis that has been proposed for incomplete protection against varicella in some individuals is that VZV antibodies have lower avidity in patients with recurrent cases (68). An absence of antibodies directed at gE, gB, and gH does not account for reinfections (69). There is considerable boost in humoral immunity to VZV upon exposure of varicella-immune subjects to patients with VZV infections (3). In one study, 32% of parents exposed to their children with varicella manifested either transient IgM antibodies or an increase in antibody titer or both (70). Whether a boost in CMI also occurs after an exposure is not known, but boosting of CMI by immunization years following natural infection has been demonstrated (4). Hope-Simpson hypothesized over 50 years ago that immunologic boosting is important for long-term maintenance of immunity to VZV and may result from either endogenous (asymptomatic reactivation of latent virus) or exogenous exposure to VZV (7). It has been demonstrated that endogenous boosting of immunity to VZV occurs (71–76).

Nosocomial Varicella

Nosocomial varicella can be a serious and expensive problem in hospitals, where both patients and employees may be susceptible to chickenpox (3). With the availability of varicella vaccine for adults and children since 1995, nosocomial varicella appears to be less of a problem in the United States than it was formerly. Because varicella-susceptible hospital employees may serve as vectors for spread of VZV to susceptible patients, it is now common to test employees for immunity to chickenpox serologically and to offer vaccine to those who are susceptible. Passive immunization (with preformed antibodies) is not useful to terminate potential nosocomial varicella outbreaks, but it is useful to protect varicella-susceptible immunocompromised patients who have been exposed to VZV (see below).

The risk of horizontal transmission of VZV in maternity wards or the newborn nursery after a hospital exposure to an adult or child is extremely low, which has been attributed to several factors (77). Many hospitalized newborn infants are placed in isolettes, and most hospital employees and mothers and their newborn infants have antibodies to VZV and are at reduced risk of developing clinical illness. Because IgG antibodies to VZV cross the placenta, newborns of immune mothers are at least partially protected. Even in low-birth-weight infants, serum antibodies to VZV are usually detectable. Nevertheless, a few episodes of transmission of varicella in the newborn nursery have been reported.

Many more instances of an absence of transmission following exposures in this setting, however, have been recorded (77).

Incidence and Prevalence of Zoster

Zoster is traditionally a disease of adults. Patients who have zoster usually have a history of a previous attack of varicella or vaccination (3, 7). Zoster is rare in childhood, but there is an increased incidence in young children who had varicella either in utero or before reaching their second birthday (77). Chickenpox in the first year of life increases the risk of childhood zoster by a relative factor between roughly 3 and 21 (3), possibly as a result of immaturity of the immune response to VZV in young infants. Latent infection is also extremely common in infants with congenital varicella syndrome; zoster may occur in over 20% of affected infants (77).

Over a lifetime, zoster occurs in approximately 30% of individuals (4). The incidence of zoster in a population begins to increase sharply at about the age of 50 years; the incidence during the sixth decade, approximately 5 per 1,000 person-years annually, is almost double that of the previous decade (7, 78). During the eighth decade the incidence more than doubles again, to 14 per 1,000 person-years annually (7). The increased incidence of zoster with advancing age is attributed to a relative loss of CMI to VZV that occurs naturally with aging (3).

Zoster occurs with increased frequency in immunocompromised patients; those who are severely immunocompromised may also develop disseminated VZV infection with viremia (79). Zoster may be particularly frequent and severe following bone marrow transplantation, after which as many as 35% of patients develop it within a year (3). Spinal trauma, irradiation, and corticosteroid therapy may also be precipitating factors. The distribution of lesions in chickenpox, which primarily involves the trunk and head, is reflected in a proportionately greater representation of these regions in the dermatomal lesions of zoster (3). Zoster may recur, either in the same dermatome or in a different dermatome; however, the chance of developing recurrent zoster seems to be lower in healthy individuals than the chances of recurrent HSV (7). The reported incidence of zoster in various groups is shown in Table 2.

Mortality

The mortality for varicella and zoster are low, but the rates increase with advancing age, pregnancy, and decreasing immunocompetence. For varicella, the reported case fatality rate is lowest in children aged 1 to 14 years (0.75 per 100,000). The reported case fatality rate in infants less than 1 year old is 6.23 per 100,000, in those aged 15 to 19 it is 2.7 per 100,000, and in those 30 to 49 years old it is 25.2 per 100,000 (80). In the prevaccine era 100 to 150 deaths were reported in the United States annually (3). Pregnancy, particularly when varicella occurs in the third trimester, may increase morbidity and mortality (3, 77, 81, 82). In a report of 44 consecutive cases of varicella during pregnancy, varicella pneumonia occurred in 9% of women, with one fatality (2.4%) (82). In a more recent collaborative study, however, involving 347 pregnant women who were consecutively monitored, there were no fatalities, although 5% developed varicella pneumonia (81). The mortality for varicella in children with leukemia receiving chemotherapy is reported as 1,000 times higher than that in healthy children (3). A fatality rate of 7% was reported for children with leukemia and chickenpox in the preantiviral drug era (79). Epidemiologic data from the 1990s suggest that the incidence of severe varicella in healthy hosts was underestimated by a factor of 5 (83). Mortality related to zoster is due primarily to pneumonia; the overall risk of fatal infection is lower than that for varicella in immunocompromised patients, less than 1% (84). Zoster also has a lower mortality than varicella in otherwise healthy middle-aged or elderly adults (3).

TABLE 2 Reported approximate incidence of zoster in various populations per 1,000 person-years of observation[a]

Adults (all ages): 2–4 (7)*
Adults, age 70–79: 11 (4)
Adults, middle aged, vaccinated against varicella: 0.9 (158)*
Children with leukemia in remission (prior varicella) 25 (157)**
Children with leukemia, vaccinated: 8 (157)**
Adults with HIV (prior varicella): 51 (3)
Children with HIV (prior varicella): 163 (121)***
Children with HIV, vaccinated: 0–3 (155)***

[a]Numbers in parentheses are reference numbers. *Trend due only to wide confidence limits; **significantly different values; ***significantly different values.

Transmission

Varicella is a highly contagious disease, particularly in the early stages of the illness; VZV is spread by the airborne route (77). It was long thought that the source of transmitted virus was the respiratory tract of infected individuals. Spread of VZV in closed communities that were attributed to exposure to an index case prior to development of rash has been occasionally reported, suggesting that respiratory transmission occurs (85). It is possible, however, that an early insignificant rash went unnoticed in such patients. Studies utilizing PCR identified VZV DNA in the nasopharynx of children during preeruptive and early varicella, although the rates have been inconsistent (3). The presence of VZV DNA, however, does not necessarily indicate the presence of infectious virus. For example, VZV DNA was recovered from room dust 2 weeks after a patient was discharged, long after the virus could remain infectious (3).

Evidence suggests that cell-free virions in vesicular skin lesions of patients with VZV are the major source of infectious virus (37). Secondary to the loss of MPRs in the superficial epidermis, high concentrations of cell-free, 200-nm particles of VZV develop in skin vesicles; these may be aerosolized and thus transmit the virus to others by the airborne route (37). Presumably airborne virus infects the respiratory tract of varicella-susceptible individuals. Clinical studies of transmission of VZV in leukemic vaccinees (86) and immunocompetent children (87, 88) have also implicated skin lesions as the source of infectious virus. There was a direct relationship between the number of skin lesions and viral transmission (87, 88). In a recent study from China, an outbreak among schoolchildren became controlled only after children with rash were excluded from school attendance (87). There is one report of transmission of VZV to others during an autopsy of a patient with varicella; obviously the virus was not spread from the respiratory tract (89).

Studies of isolation of VZV from patients with varicella also implicate the skin as the source of infectious virus rather than the respiratory tract. VZV is readily isolated in cell culture from skin lesions, but it is extremely difficult to isolate the virus from the respiratory tract. Searches for infectious VZV in throat secretions of immunocompetent patients during the incubation period of varicella have

proved largely negative (90–92). There is, however, one report of isolation of VZV from nasal swabs from 3 of 23 (13%) children and from pharyngeal swabs from 2 of 22 (9%) children on days 2 and 3 after the onset of rash (92). It is possible that there may have been vesicles on the mucous membranes in these children, and also it is not clear whether any of them were immunocompromised. In contrast, VZV can readily be cultured from vesicular lesions in patients with clinical varicella. Isolation of VZV was successful in 23 of 25 (92%) cases when vesicle fluid was cultured within 3 days after the onset of rash in one study (90) and in 12 of 17 (71%) cases in another study (92). Vesicular fluid specimens collected from 4 to 8 days after onset, however, yielded VZV in only one of seven instances (90). Infectious VZV may persist longer in vesicles of zoster patients (3). In general it may be conservatively presumed that varicella is transmissible to others from 1 to 2 days prior to the onset of rash and during the first few days of rash. Skin lesions in either varicella or zoster are unlikely to harbor infectious virus once they are dry. Taken together, these observations suggest that VZV spreads predominately from skin lesions and that within several days after the onset of rash, patients with varicella are no longer contagious to others. In contrast, zoster may be infectious for a somewhat longer period especially when vesicles in older patients are slow to heal.

Zoster is not transmissible to others as such, but the vesicular lesions contain infectious VZV and are therefore potentially contagious to others as varicella is (3). Even patients with localized zoster may transmit VZV to varicella-susceptible individuals, and therefore these patients should be isolated when hospitalized. Whether VZV is spread from the respiratory tract in zoster is unknown, but it seems unlikely. Based on scant information regarding household transmission of VZV from zoster patients to varicella-susceptible contacts, zoster was estimated to be only about half as contagious as varicella (3).

PATHOGENESIS IN HUMANS

Incubation Period, Patterns of Virus Replication, and Factors in Disease Production

The incubation period of varicella ranges from 10 to 23 days, with an average of 14 days (3). During the incubation period, VZV is thought to spread to regional lymph nodes, undergo multiplication, and cause a primary low-grade viremia about 5 days later (93) (Figure 4).

In varicella, two viremic phases have been hypothesized to occur, based on studies with the virus that causes mousepox (93). Following a putative early viremia that spreads the virus to the reticuloendothelial system, there is local replication of VZV. This results in the second and greater viremia, which delivers the virus to the skin; there it causes the characteristic skin rash, in which ``crops'' of lesions develop, possibly reflecting several viremic episodes (93) (Figure 4).

A somewhat alternative proposal for varicella pathogenesis is based in part on studies of mice with severe combined immunodeficiency disease engrafted with various human tissues (SCID hu mouse model) infected with VZV (94). Following exposure of susceptible contacts, VZV infects tonsillar T cells as cell-free virus and multiplies in the respiratory mucosa, where T lymphocytes are infected. In VZV-infected SCID hu mice, these T cells deliver VZV promptly to implanted skin. In infected individuals, only limited viral multiplication in the skin is thought to take place at first, as a result of the innate immune response. Innate immunity is eventually overcome as the incubation

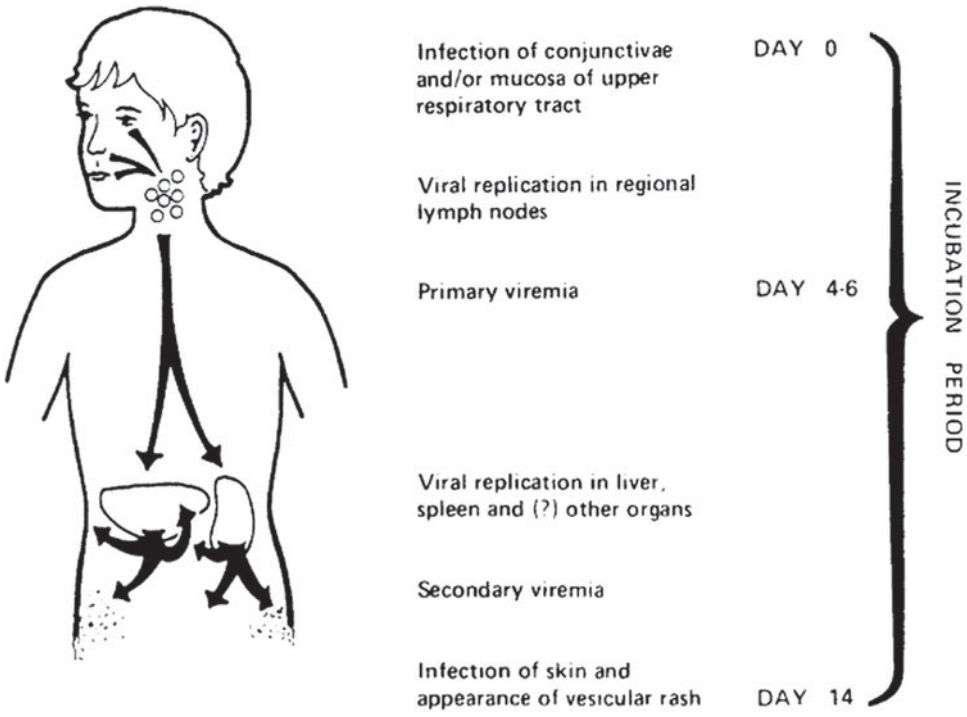

FIGURE 4 Diagram showing proposed pathogenesis of varicella regarding events during the incubation period. (Reprinted from reference 93 with permission.)

period of the disease comes to an end. Because of circulation of infected T cells, there is a low-grade viremia for a week or so after infection, but as more T cells and keratinocytes are infected, the viremia increases, innate immunity is overcome, and the rash develops. At that time adaptive immunity develops, and within a week or so the pathogen is controlled and the patient recovers. VZV has been isolated from blood cultures either a few days before onset of rash or within 1 to 2 days after rash onset in immunocompetent children (95). VZV has also been isolated from blood obtained from immunocompromised patients with varicella or zoster (3). Experiments in SCID hu mice and human skin cellular implants indicate that human CD4 and CD8 lymphocytes are infected with VZV (96).

A number of strategies for immune evasion of VZV by host cells that facilitate VZV multiplication have been described, some unique to this herpesvirus (97–100). For example, in infected fibroblasts, VZV down-regulates major histocompatibility complex (MHC) class I antigens and thereby interferes with antigen presentation to cytotoxic T cells. Additionally, MHC class II expression can be blocked by VZV gene products, resulting in a decreased gamma interferon response. Immune evasion is thought to play an important role, particularly in viral multiplication that occurs during the incubation period of varicella.

The skin lesions of varicella begin as macules and progress rapidly to papules, vesicles, pustules, and scabs. Vesicles are located in the superficial epidermis. With time, the fluid changes from clear to cloudy, as polymorphonuclear leukocytes (PMNs) reach the site. Interferon is present in vesicular fluid, reflecting the adaptive cell-mediated immune response of the host (101). The predominant cell in vesicular lesions is the PMN, which may play a role in generating interferon in vesicular lesions and play a role in recovery (3). *In vitro* data also suggest that the PMNs are involved in host defense against VZV, possibly by mediating antibody-dependent cellular cytotoxicity (ADCC) (3). Natural killer (NK) cells also play a role in host defense against VZV (8). Cytotoxic CD4 and CD8 lymphocytes, however, represent the major host response that controls VZV infection (3).

Histologic changes in the skin vesicles are similar for chickenpox and zoster. The hallmarks of each are multinucleated giant cells and intranuclear inclusions. In varicella these are primarily localized in the epidermis, where ballooning degeneration of cells in the deeper layers is accompanied by intercellular edema. As edema progresses, the cornified layers and basal layers separate to form a thin-roofed vesicle. An exudate of mononuclear cells is seen in the dermis without characteristic nuclear changes of epithelial cells in this region (Figure 5) (102).

In zoster, in addition to skin lesions that resemble those of varicella, the dorsal root ganglion of the affected dermatome exhibits a mononuclear inflammatory infiltrate. There may also be necrosis of ganglion cells and demyelination of the corresponding axon (103).

Latent VZV is present in human sensory dorsal root and cranial nerve ganglia in human autopsy specimens in persons who have had varicella. Prior to development of zoster, VZV reactivates in the neuron, resulting in lytic infection, and then it travels down the affected sensory nerves to the skin, causing a characteristic unilateral vesicular rash (Figure 6). Zoster may last for several weeks, particularly in immunocompromised patients, who not only are at increased risk of developing this disease as a result of low levels of CMI to VZV but also may require long times to recover for the same reason.

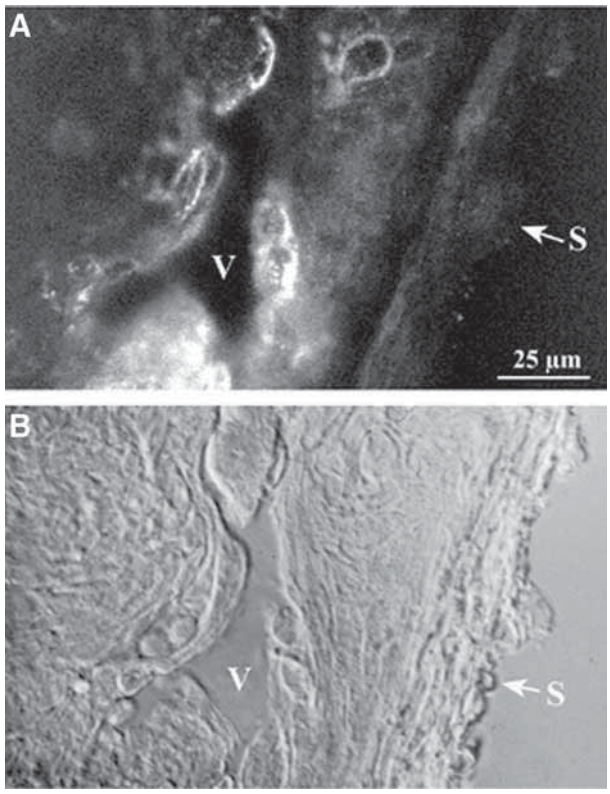

FIGURE 5 Skin vesicle from a patient in the early stages of varicella. The vesicle has not been unroofed. (A) The specimen was stained with fluorescein-tagged monoclonal antibodies to gE of VZV. (B) Same specimen as in panel A, viewed by Nomarski interference contrast microscopy. V, vesicular space; S, outer surface of epidermis.

Immune Responses

Both humoral and cell-mediated adaptive immune responses to VZV develop within a few days after onset of varicella. Peak antibody levels are attained after 4 to 8 weeks, remain high for about 6 months, and then decline. IgG antibody to VZV is detected in healthy adults for decades after varicella (3). After active immunization against varicella, antibody titers are lower than after natural infection but may persist for periods of 20 years or more (104). Because doses of varicella vaccine used in different countries and made by different companies vary in composition and dosage, persistence of antibodies after vaccination may vary in different locations. Serum IgG, IgA, and IgM develop after both varicella and zoster. Zoster occurs in the face of high levels of specific antibodies, but significantly higher titers develop during convalescence, reflecting the anamnestic response to this reactivated infection (3).

CMI plays the major role in host defense against VZV, probably because the virus spreads almost entirely in the body as an intracellular pathogen. CMI can be demonstrated by stimulation of lymphocytes *in vitro* with VZV antigens (105, 106), by an intradermal skin test (66), and by specific lysis of histocompatible target cells by cytotoxic T cells (107). Natural killer (NK) cells and antibody-dependent cell-mediated cytotoxicity (ADCC) against VZV have also been described (108). CMI reactions can be detected for years after varicella, although this response may wane in many individuals after age 50 (109).

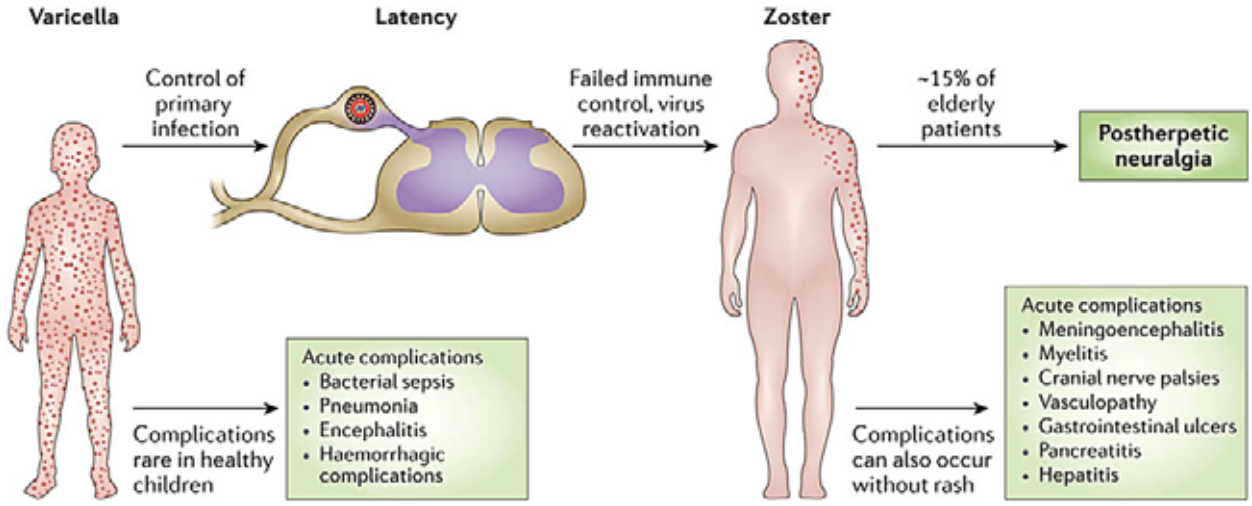

FIGURE 6 Proposed pathogenesis of zoster. (Reprinted from reference 180 with permission.)

Exactly how immunity to varicella and zoster is mediated, however, is still unclear. It is generally agreed that CMI, in the form of T-cell cytotoxicity, is more important than humoral immunity in recovery from infection. For example, children with isolated agammaglobulinemia are not at increased risk of developing severe varicella. CMI is probably crucial during chickenpox because spread of VZV within the body is almost exclusively by the intracellular route, rather than by release of cell-free virus, as occurs in vesicular fluid.

The response that prevents clinical illness after reinfection with VZV is not known for certain, although it is presumed to be some form of CMI, perhaps at times in concert with antibodies. Patients with agammaglobulinemia are not subject to recurrent varicella. On the other hand, elderly adults, who often have low CMI or an absence of CMI to VZV, are not particularly subject to second attacks of varicella; presumably, their antibodies protect them from recurrent chickenpox. Specific antibodies may play a role in immunity because passive immunization can be used to prevent or modify varicella in exposed susceptible individuals. It may be that antibodies are particularly effective right after infection occurs because of a brief period of multiplication in tonsils in which cell-free VZV, which can be neutralized, is produced. It is thus possible that passive immunization lowers the initial viral load, resulting in modified or asymptomatic infection. Certain antibodies, particularly those to gH, moreover, may impede cell-to-cell spread of the virus (30). Young infants, however, can develop varicella after exposure despite detectable transplacental antibody titers, but usually the illness is attenuated (77). Vaccinated leukemic children have developed modified cases of breakthrough varicella despite detectable VZV antibodies or positive CMI responses as measured by lymphocyte stimulation at exposure to VZV, but they do not have normal immune systems (3). They are, however, highly likely to be protected if they have both humoral and cellular immunity to VZV at exposure (110). Healthy individuals with detectable humoral immunity at exposure to VZV are far less likely to develop clinical illness than those lacking these responses (111). Both antibodies and CMI seem to participate in various aspects of protection against VZV, suggesting that redundancy in the adaptive immune response may provide the best strategy against the virus for the host.

VARICELLA

Clinical Manifestations

Varicella is a highly contagious, usually self-limited systemic infection, characterized by fever and a generalized pruritic rash, lasting about 5 days (Figure 7). A prodromal phase in children is unusual, but it is not uncommon for adults to experience a prodrome of malaise and fever for 1 to 2 days prior to onset of the rash (3). The rash is characteristically more concentrated on the trunk and head than on the extremities, and it typically evolves as a series of "crops" over 1 to 2 days in healthy hosts. Most children with varicella develop from 250 to 500 skin lesions, many of which are vesicular (65). In many cases a few lesions may develop in the mouth or conjunctiva or on other mucosal sites. Rarely, the skin lesions may be bullous or hemorrhagic. Residual scarring is exceptional, but depigmented areas of skin may result and be persistent. Constitutional symptoms may be mild despite an extensive exanthem, but usually the extent of rash reflects the severity of the illness. It is not uncommon to observe a transient increase in hepatic aminotransferase levels without jaundice during varicella, but this is not considered a complication of the disease (3). Adults are more likely to develop severe varicella than children, presumably because of lower CMI responses to VZV than in children (3). Newborn infants who acquire varicella from their mothers in the few days prior to delivery are also at risk of developing severe varicella, presumably because of the immature CMI response in very young babies in the absence of specific maternal antibodies (3, 77). Patients with various immunodeficiencies affecting the CMI response to VZV (T lymphocytes and NK cells) (112) and those receiving high doses of steroids for any reason are at risk to develop severe varicella (3, 8).

Clinical Diagnosis

It is usually not difficult to make a clinical diagnosis of varicella because the vesicular pruritic rash is so characteristic. In questionable cases, epidemiologic information may be useful, such as a history of recent exposure to varicella or zoster and subsequent transmission of varicella to another person. The differential diagnosis of varicella includes generalized HSV infection, rickettsial pox, impetigo, allergic reactions (including Stevens-Johnson syndrome), poison

FIGURE 7 Varicella on day 5 in an otherwise healthy 2-year-old boy, showing the typical distribution of skin lesions.

ivy, and insect bites. In locations where vaccine is being used, laboratory confirmation of mild breakthrough varicella in an immunized child (see below) may be required.

Complications

The most frequent complication of varicella in healthy hosts is bacterial superinfection of the skin, lungs, or bones. Severe morbidity and even mortality involving group A beta-hemolytic streptococcal superinfections have been reported (113). Central nervous system complications that may precede or follow varicella in healthy hosts include transient cerebellar ataxia, a severe form of cerebral encephalitis, aseptic meningitis, transverse myelitis, and stroke. Because aspirin is no longer recommended for children with varicella, encephalopathy due to Reye syndrome has become rare. Meningoencephalitis and cerebellar ataxia, which usually occur between 2 and 6 days after onset of the rash, may also occur during the incubation period (3, 114). With these complications, the cerebrospinal fluid (CSF) may be normal or may exhibit a mild lymphocytic pleocytosis (< 100 cells/ml), a moderate elevation of protein (< 200 mg), and normal glucose. VZV encephalitis can be life threatening; symptoms may reverse rapidly or gradually improve. Chronic neurologic or developmental sequelae may occur (115). Cerebellar ataxia may persist for days to weeks but is almost invariably self-limited. Strokes after varicella are unusual, have only recently been recognized as a complication of the infection, and are hypothesized to be secondary to vasculitis provoked by VZV (116). Varicella may rarely

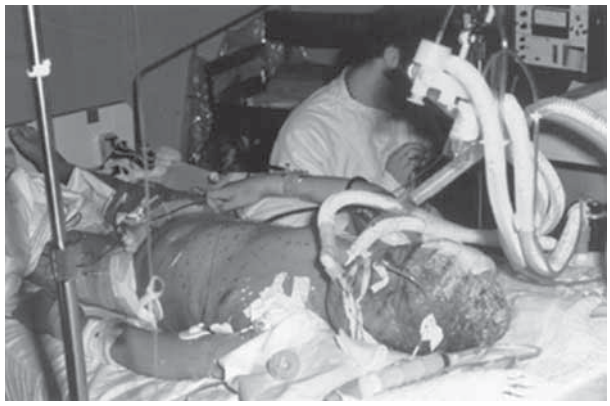

FIGURE 8 Hemorrhagic fatal varicella in a child with underlying lymphoma.

cause neutropenia and thrombocytopenia with hemorrhagic complications 1 to 2 weeks after the initial infection (117). Arthritis is an infrequent, transient complication; VZV has on occasion been isolated from joint fluid. Vesicular lesions that involve the eyelids and conjunctivae rarely cause serious ocular complications (118). Other rare complications of varicella include renal failure, myocarditis, pericarditis, pancreatitis, orchitis, and purpura fulminans (3, 102, 119).

Varicella may be severe and even fatal in immunocompromised patients. These include persons with an underlying malignancy; congenital or acquired deficits in CMI, such as those who have undergone organ transplantation or have underlying human immunodeficiency virus (HIV) infection; and individuals receiving high doses of corticosteroids for any reason (3). Patients with deficiencies in CMI may manifest progressive varicella, with continuing fever and development of new vesicular lesions for as long as 2 weeks (Figure 8). Their characteristic skin lesions are often large, umbilicated, and hemorrhagic; primary varicella pneumonia is a frequent complication. Alternatively, some immunocompromised patients develop an acute form of varicella with disseminated intravascular coagulation that is rapidly fatal (3). A visceral disease rate of 30% and a fatality rate of 7% were reported for leukemic children who developed chickenpox prior to the availability of antiviral drugs (120). Severe varicella has been observed in children with underlying infection with HIV, especially those who have clinical evidence of AIDS, but most HIV-infected children develop mild to moderate forms of varicella. The illness in this population is not potentially as severe as in children with leukemia (121). However, because it is difficult to predict in advance whether a child with HIV infection will develop severe varicella, most physicians routinely elect to treat them at onset with antiviral drugs. Varicella does not seem to be a cofactor for clinical progression of HIV infection to AIDS, but chronic infections with wart-like hyperkeratotic lesions with occasional virus dissemination may develop in these patients (122) (Figure 9). These lesions appear to be a persistent form of VZV infection rather than classic zoster.

Primary varicella pneumonia accounts for many of the fatalities ascribed to varicella (Figure 10). It most commonly occurs in immunocompromised patients, adults, and neonates with chickenpox (3). In a chest X ray study of military recruits with varicella, radiographic evidence of pneumonia was found in 16%, although only one quarter of these

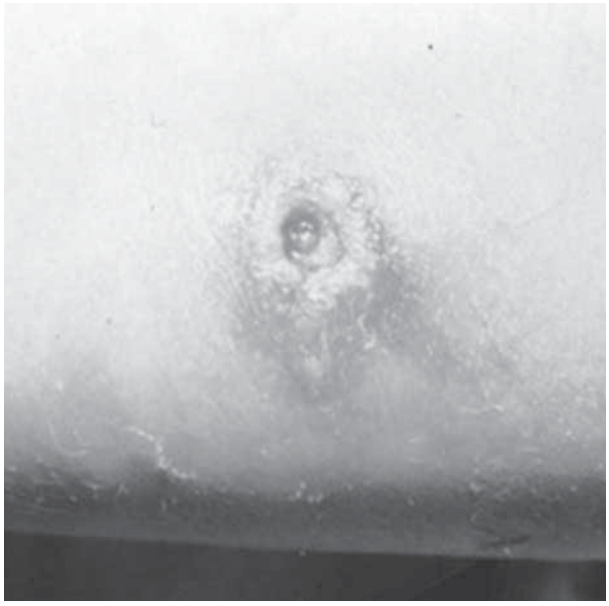

FIGURE 9 Chronic VZV skin lesion in a child with underlying HIV infection.

patients had pulmonary symptoms (123). Pneumonia usually occurs within several days after the onset of rash, but sometimes this interval may be longer. Symptoms include fever and cough in almost all cases and dyspnea in more than 70%. Other common symptoms and signs include cyanosis, rales, hemoptysis, and chest pain. The chest X ray typically reveals a diffuse nodular or miliary pattern, most pronounced in the perihilar regions (124). The availability of antiviral chemotherapy has greatly improved the outcome of varicella pneumonia.

ZOSTER

Clinical Manifestations

Zoster usually begins as a localized skin eruption involving one to three dermatomal segments. The characteristic distribution of the unilateral rash reflects its pathogenesis, reactivation of latent VZV from dorsal root or cranial nerve ganglia (Figure 11). The skin lesions resemble those of varicella but tend more toward confluence. Zoster usually develops in patients who are at least somewhat immunodeficient, as a result of disease, chemotherapy, radiotherapy or normal aging. Healthy young adults who are not immunocompromised may develop zoster, presumably resulting from a transient decrease in CMI to VZV as a response to a stimulus such as another viral infection or stress. Because many immunocompromised persons do not develop zoster, however, deficiency of CMI to VZV is thought to be a necessary but not sufficient to develop this illness. It is likely that reactivation of VZV first occurs and that clinical zoster then results when the CMI response is deficient.

There is a spectrum of clinical manifestations of zoster. Zoster with no rash but with dermatomal pain has been described (zoster sine herpete) (125, 126), as has visceral zoster without skin rash (125). Increases in VZV antibody titer in the absence of an exposure to the virus have been ascribed to silent reactivation of VZV (3). Subclinical viremia in patients after bone marrow transplantation has been demonstrated by PCR (75). Shedding and transmission of infectious VZV from asymptomatic individuals has not been documented as it has for HSV. Asymptomatic reactivation of VZV following extreme stress has been detected (127, 128).

Clinical Diagnosis

It is usually not difficult to make a clinical diagnosis of zoster because the unilateral dermatomal rash is so characteristic. In one study, however, 13% of cases clinically diagnosed as zoster were proven by culture to be due to HSV infection (129). HSV in particular should be considered in the differential diagnosis when the rash has a trigeminal/maxillary, breast (T4), or sacral distribution, and especially if the rash is recurrent. The unilateral rash of zoster most often involves the thoracic and cervical areas, followed by the face. Laboratory verification is becoming of greater importance in the vaccine era, when it may be of clinical interest to determine if a zosteriform rash is due to VZV and if it is wild or vaccine type.

Self-limited meningitis indicated by abnormalities of the CSF, including pleocytosis (predominantly of mononuclear cells) and elevated protein, may develop in many patients with zoster (3). Complete healing of the rash usually occurs within 2 weeks but may require 4 to 6 weeks, especially in immunocompromised patients. Segmental pain and/or itching are common symptoms in zoster. These sensations may precede the onset of rash. Other causes of acute segmental pain such as myocardial infarction, acute abdomen,

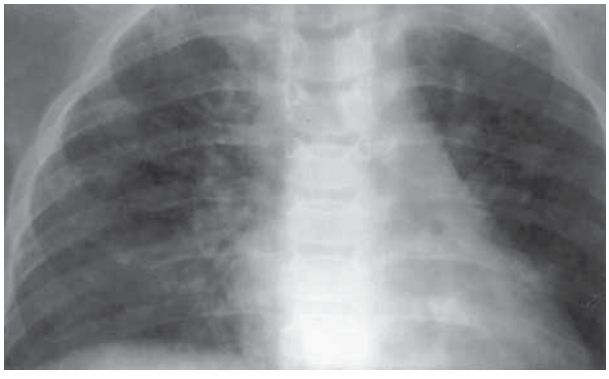

FIGURE 10 Chest radiograph of a patient with primary varicella pneumonia. (Courtesy of Walter Berdon.)

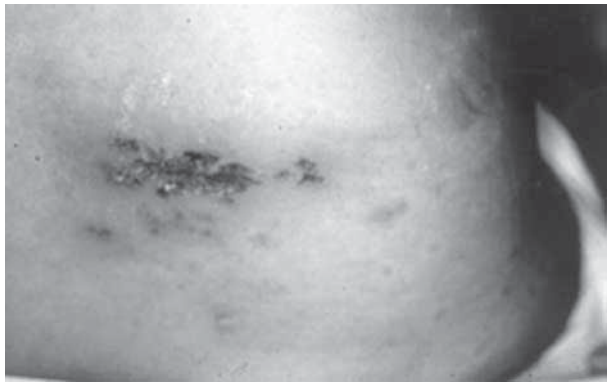

FIGURE 11 Zoster in a 10-year-old boy with underlying leukemia, showing the unilateral lumbar distribution of the skin lesions.

and pleurisy may need to be excluded in patients with preeruptive zoster pain or zoster sine herpete.

Complications

From 25% to 50% of persons over the age of 50 who develop zoster may develop protracted pain, or postherpetic neuralgia (PHN), following healing of the rash. Treatment with antiviral drugs has not led to a decrease in the incidence of PHN, although therapy may decrease the duration of pain (3). Pain may persist for months to years and is described as aching, jabbing, or burning. Abnormal sensations may also occur, such as pain after a minimal nonpainful stimulus (allodynia) and severe pain after a mild pain stimulus (hyperalgesia). The precise cause is unknown. Hypotheses include an aberrant immune response to the virus in the neuron, pain resulting from repair of neuronal damage, and continuing low-level multiplication of virus in the ganglion, with nerve damage from inflammation and hemorrhage. Evidence against the last hypothesis is the observed usual failure of antiviral therapy to relieve PHN. Both the incidence and duration of PHN are directly related to increasing age (4, 130).

Zoster can also involve various cranial nerves. Zoster in the ophthalmic branch of the trigeminal (fifth) nerve may cause dendritic keratitis, anterior uveitis, iridocyclitis with secondary glaucoma, and panophthalmitis (131). Retinitis from VZV is a particular problem for HIV-infected persons (132). Reactivation of VZV involving the maxillary branch results in oral lesions. Motor nerve deficits may also be associated with zoster of the trigeminal nerve. Reactivation in the geniculate ganglion of the seventh (facial) cranial nerve and the eighth (auditory) cranial nerve, termed the Ramsay Hunt syndrome, is associated with facial palsy.

Motor deficits occur in approximately 1% of zoster cases (133). Bladder dysfunction or ileus with intestinal obstruction is an unusual complication of lumbosacral zoster. Partial paralysis of an extremity can also occur after zoster. The prognosis for recovery from these motor deficits is good, although residua may follow in as many as 15% of patients (133). Transverse myelitis is an extremely rare complication with a high mortality (130).

Zoster is complicated by clinical encephalitis in an estimated 0.2% to 0.5% of cases. Risk factors include advancing age and cranial nerve involvement. Symptoms, which often occur about a week after onset, include altered state of consciousness, headache, photophobia, and meningismus. The average duration of encephalitic symptoms is about 2 weeks (130, 133). Neurologic sequelae may follow recovery.

Granulomatous angiitis resulting in signs and symptoms resembling those of a cerebrovascular hemorrhage or thrombosis (stroke) is an unusual complication of zoster that occurs particularly in the elderly (130). The central nervous system symptoms characteristically occur on the side opposite the rash, and they may develop months after onset of the rash. There is a high mortality, and at autopsy, vascular inflammation with thrombosis and microinfarcts are seen in the temporal and other arteries (130, 134–136).

A syndrome of zoster accompanied by inappropriate secretion of antidiuretic hormone has been described to occur in immunocompromised patients. It may be associated with severe abdominal pain and is associated with a poor prognosis (137, 138).

Visceral zoster with predominant abdominal symptoms, such as pain and even gastrointestinal obstruction, has been described, particularly for immunocompromised patients. Pain may precede the rash by several days, and in some cases no rash develops (139). Latent VZV in the enteric nervous system of humans has been described, and its consequences (such as causing gastric ulcers resulting from reactivation of VZV in the absence of rash) are being identified (43, 123).

CONGENITAL VARICELLA SYNDROME

Congenital varicella syndrome has a characteristic constellation of developmental abnormalities and was first described in case reports of infants born to mothers who had varicella in early pregnancy. In 1947, LaForet and Lynch described an infant with multiple congenital anomalies after maternal chickenpox during the 8th week of pregnancy (140). The infant had hypoplasia of the right lower extremity with clubfoot and an absence of deep tendon reflexes, cerebral cortical atrophy, cerebellar aplasia, chorioretinitis, torticollis, insufficiency of the anal and vesical sphincters, and scarred cutaneous lesions of the left lower extremity. While initially it was thought that the syndrome occurred after maternal VZV infection in the first trimester of pregnancy, an equal number of reported cases have occurred following maternal varicella in the second trimester. Over 100 affected infants have now been reported (77). Almost all of these cases have followed maternal varicella; a few cases following maternal zoster have been described but rarely substantiated (3). If a woman develops varicella in the first or second trimester of pregnancy, it is estimated that there is a 2% chance that her baby will be affected by this syndrome. In the prevaccine era it was estimated that about 40 infants were born annually in the United States with this syndrome (38). Scars of the skin, usually described as cicatricial, are the most prominent stigmata, reported in 70% of cases (Table 3) (77). Other frequent abnormalities include those involving the eyes, such as chorioretinitis, microphthalmia, Horner syndrome, cataract, and nystagmus. Hypoplastic limbs, cortical atrophy and/or mental retardation, and early death are also commonly observed.

TABLE 3 Clinical data on over 100 reported infants with developmental defects whose mothers had VZV infections (over 95% with varicella) during the first or second trimester of pregnancy between 1947 and 2002[a]

Defect	% of infants with defect
Skin scars	70[b]
Eye abnormalities (chorioretinitis, Horner syndrome/anisocoria, (microphthalmia, cataract, nystagmus)	60
Neurologic abnormalities (retardation, seizures, paralysis)	60
Abnormal limb[c] (hypoplasia, equinovarus, abnormal/absent digits)	50
Prematurity, low birth weight	35
Early death	25
Zoster in infancy	20

[a]Modified from reference 77 with permission of Elsevier.
[b]Cicatricial in over 60%.
[c]Of infants with hypoplastic limb 50% had mental retardation.

LABORATORY DIAGNOSIS

The diagnosis of VZV infection can usually be made clinically, but laboratory diagnosis may be necessary in unusual cases, particularly for vaccinated individuals who develop either possible varicella or zoster. Laboratory diagnosis of active VZV infection is facilitated by the presence of VZV in superficial skin lesions, where it is accessible for testing. Diagnosis is best made by demonstration of VZV DNA or specific viral antigens in vesicular skin lesions. PCR has supplanted viral culture as a diagnostic tool; it has been successfully employed for diagnosis of VZV utilizing skin scrapings, vesicular fluid, respiratory secretions, and CSF (64). PCR can also be used to differentiate between vaccine and wild-type VZV (13) and to demonstrate susceptibility to antiviral drugs (141). VZV antigen may be demonstrated by immunofluorescence (IF), using a commercially available monoclonal antibody to gE of VZV that is conjugated to fluorescein (64). This is a highly sensitive and rapid diagnostic method that can be completed within about an hour and is therefore clinically useful.

The presence of VZV by IF or culture in clinical material obtained from skin or other lesions, or autopsy tissue, is diagnostic of an active infection (142) because unlike for HSV, there is no known infectious carrier state in asymptomatic individuals. The presence of VZV DNA in specimens such as nervous tissue, however, suggests but does not prove latent VZV infection.

If a culture is to be successfully performed, vesicular fluid should be obtained as early in the course of illness as possible to facilitate isolation of VZV. Within several days after onset of varicella, vesicular fluid is no longer likely to be infectious, although viable VZV may be present in zoster lesions for a longer period, especially in immunocompromised patients. Isolation of VZV is a relatively slow method, since it takes at least 48 hours before the first signs of viral CPE are seen. It is also less sensitive than IF staining, since infectious virus persists for a shorter length of time in vesicles and is more labile than viral antigens. PCR is the most sensitive technique (143). VZV is rarely isolated from CSF, throat, pharyngeal, and conjunctival specimens. Human lung fibroblasts such as WI-38 cells are most frequently utilized to isolate VZV in tissue culture. The Tzanck test provides only nonspecific information; it has been replaced by more sensitive and specific methods mentioned previously.

A number of sensitive serologic tests are available to measure antibodies to VZV. These include the fluorescent antibody to membrane antigen (FAMA) method, latex agglutination, and enzyme-linked immunosorbent assay (64). Antibody to VZV develops within a few days after onset of varicella, persists for many years, and is present before the onset of zoster. VZV infections may be documented by a ≥4-fold rise in VZV antibody titer in acute- and convalescent-phase serum specimens. The presence of specific IgM in one serum specimen suggests but does not necessarily prove recent VZV infection, either varicella or zoster (64). Persistence of VZV antibody in infants beyond 8 months of age is highly suggestive of intrauterine varicella (144). Immunity to varicella is highly likely to be present if a positive titer of antibody (measured by a reliable assay) to VZV is demonstrated with a single serum sample from a child or an adult with no history of disease. Serologic methods, however, particularly commercial enzyme-linked immunosorbent assays, may fail to identify many individuals who have been immunized with live attenuated varicella vaccine (64). While the FAMA assay is the most sensitive test for this purpose, it is not generally available.

The value of serologic procedures for diagnosis of zoster is limited, since heterologous increases in titer of antibody to VZV may occur in patients with HSV infections who have previously had varicella. This phenomenon has been ascribed to antigens common to the two viruses (64).

PREVENTION

VZV is such an infectious agent that general measures are not very useful for prevention of varicella in susceptible individuals. Some protection for hospitalized patients, however, can be accomplished by isolation of patients with moist VZV skin lesions, particularly if they are placed in rooms with negative-pressure ventilation. Hospitalized patients with active VZV infections should be admitted to a private room, and hospital personnel and visitors should wash their hands before and after entering the room and wear masks, gowns, and gloves while in it. Spread of VZV by fomites does not occur. Children with obvious varicella should be excluded from school until the skin lesions are dry. Vaccinated children who develop a few skin vesicles between 2 and 6 weeks after vaccination are unlikely to spread VZV and can attend school if they are otherwise well. Vaccinees with rash 1 to 14 days after immunization are likely to be infected with wild-type VZV. In recently vaccinated patients the development of more than 50 vesicles is highly suggestive of wild-type infection, which unlike the Oka strain, is highly transmissible. These patients, therefore, should be excluded from school and if hospitalized should be isolated from other patients.

Prevention of varicella may be accomplished with either active or passive immunization. Varicella-susceptible children at high risk of developing severe chickenpox should be passively immunized if they are closely exposed to someone with either varicella or zoster. Passive immunization usually modifies varicella but may prevent it. Passive immunization may be a life-saving measure for a high-risk susceptible child.

Passive Immunization against Varicella

Varicella-zoster immunoglobulin (VZIG) was approved by the Food and Drug Administration (FDA) for use in 1981. Because the demand for this product decreased sharply after licensure of varicella vaccine in 1995, it is no longer being produced in the United States. It was shown to be most effective when administered up to 3 days after exposure and perhaps helpful for as long as up to 5 days. VZIG was replaced by a new varicella immunoglobulin product (VariZIG), manufactured in Canada (telephone numbers for information are 800-843-7477 and 800-746-6273). The dose is 1.25 ml (one vial or 125 U) for each 10 kg of body weight, with a maximum dose of 6 ml (five vials or 625 U) intramuscularly.

The major use of passive immunization is for prevention of severe varicella in people (usually children) who have been closely exposed to varicella or zoster and are at high risk of developing severe or fatal chickenpox. This includes immunocompromised patients and also newborn infants whose mothers have active varicella at the time of delivery. Passive immunization is customarily readministered to high-risk susceptible persons who are closely reexposed to varicella or zoster 3 weeks following a first exposure for which passive immunization was given.

Infants and children who are listed in the high-risk groups should be considered to be susceptible to varicella if they have no history of having had chickenpox. It is potentially hazardous to withhold passive immunization in an immunocompromised child who based on a positive antibody titer has no history of previous clinical varicella because of the possibility of a false-positive antibody test. Only about 25% of adults with no history of varicella are truly susceptible; therefore, passive immunization should generally be administered to VZV-exposed adults only if they are serologically proven to be susceptible to chickenpox. Presumably, passive immunization is useful for adults, but in contrast to use in children, for whom efficacy has been established, no efficacy studies have been performed in adults. An alternative for healthy varicella-susceptible adults with a known exposure to VZV would be to institute oral antiviral therapy at the very first sign of clinical varicella. The latter approach is considerably less expensive than passive immunization. Susceptible adults who do not develop varicella after a close exposure should be strongly considered for active immunization against varicella (see below).

Patients with HIV infection, especially those with AIDS, are at increased risk of developing severe varicella (3, 77). Their management should be similar to that of other varicella-susceptible immunocompromised children and adults.

Infants whose mothers have active varicella at delivery should receive passive immunization; specifically, this includes infants whose mothers have onset of chickenpox 5 days or less prior to delivery or within 48 hours after delivery. The transplacental route of infection and the immaturity of the immune system probably account for the severity of varicella in these infants (77). The dose of VZIG or VariZIG for newborn infants is 125 U intramuscularly. Attack rates for varicella of up to 50% in infants exposed to mothers who have varicella have been reported, despite administration of VZIG (77). Usually varicella is mild in passively immunized infants; severe varicella after timely administration of VZIG is rare (3). Passively immunized infants should be monitored carefully, but usually these infants can be managed on an outpatient basis even if they develop varicella. They may be given therapeutic oral acyclovir (ACV). The rare passively immunized exposed infant with varicella who develops an extensive skin rash (more than 100 vesicles) or possible pneumonia should be hospitalized and treated with intravenous ACV.

It is unnecessary to passively immunize full-term infants who are exposed to VZV after they are 48 hours old. Passive immunization is optional for newborn infants (under 1 week old) if their siblings at home have active varicella, particularly if the mother has no history of chickenpox or if the mother develops varicella. Infants whose mothers have previously had varicella and who are exposed to siblings or others with chickenpox are unlikely to develop severe varicella because they are at least partially protected by transplacental maternal antibody. Infants exposed to mothers with zoster do not require passive immunization because they and their mothers have high titers of antibodies to VZV. Some low-birth-weight infants may have undetectable VZV antibody titers at birth; therefore, it is recommended that newborn infants weighing less than 1,000 g or who are of less than 28 weeks' gestation who are exposed to VZV be passively immunized, even if their mothers had varicella.

Zoster occurs despite humoral immunity to VZV, and patients with zoster have very rapid rises in VZV antibodies; passive immunization is thus not useful to treat or prevent zoster. It is not known whether passive immunization of pregnant varicella-susceptible women who have been closely exposed to VZV will protect the fetus against the congenital varicella syndrome. Passive immunization is potentially useful to prevent severe varicella in varicella-susceptible pregnant women who are exposed to VZV.

Alternatives to VariZIG include intravenous immunoglobulin, 400 mg/kg, and zoster immune plasma (3).

Active Immunization against Varicella

Active immunization against varicella can be successfully accomplished with live attenuated varicella vaccine. This vaccine was developed in Japan in 1974 (145) and was approved by the FDA in the United States in March 1995 for universal administration to healthy children and adults who are susceptible to varicella (146). The vaccine is well tolerated and safe (3). The major complication following vaccination of healthy persons is a vaccine-associated rash, which occurs in 5% of vaccinees about 1 month later (3). These rashes are usually extremely mild in healthy vaccinees, with an average of five skin lesions that are generalized and/or at the injection site. The risk of transmission of Oka vaccine virus to others is present only while the vaccinee has rash, is exceedingly rare (1 transmission per 10 million vaccinees), and is much lower than the rate of transmission after exposure to wild-type VZV (3). The risk to anyone from wild-type VZV is greater than the risk from vaccine-type VZV. Therefore, it is recommended to immunize hospital workers and individuals whose family members are immunocompromised or pregnant, especially if they are susceptible to varicella (146). Highly immunocompromised individuals inadvertently closely exposed to a vaccinee with rash can be passively immunized if they are susceptible to varicella, although this is not considered mandatory.

Vaccinated individuals who develop a rash within 2 to 3 weeks after immunization may have been exposed to wild-type VZV prior to being vaccinated and are likely to have a rash caused by the wild-type virus (44). This should especially be suspected if many lesions occur and are accompanied by systemic symptoms, which are unlikely to be caused by the vaccine-type virus. It is extremely helpful in trying to evaluate such a situation to utilize PCR to differentiate between wild-type and vaccine-type VZV (3).

Live attenuated varicella vaccine is highly protective against chickenpox in healthy and immunocompromised children and in healthy adults. Not all vaccinees, however, are completely protected, particularly after one dose of vaccine (3). Roughly 15% of children may manifest a modified illness, referred to as breakthrough varicella, months to years after receipt of one dose. Varicella vaccine has, however, been over 95% effective in preventing severe varicella (3). Varicella vaccine is less effective in adults than in children; even after two doses of vaccine, only 70% of adults achieve complete protection from varicella following a close exposure to VZV, although the remainder usually have partial protection (147). In contrast, 85% of immunized healthy children are protected after only one dose of vaccine (3, 143, 148). Breakthrough varicella is almost always a modified illness, with fewer than 50 skin lesions and minimal systemic signs. In one study, about two thirds of cases of breakthrough varicella were classified as mild (< 50 skin lesions) (148).

In order to improve protection against breakthrough varicella in healthy child vaccinees, a second routine dose of vaccine was recommended by the Centers for Disease Control and Prevention (CDC) and the American Academy of Pediatrics in June 2006 (146). The second dose may

be administered at any time after 1 month following the first dose, but an interval of at least 3 months is preferred. Varicella vaccine for children may be given either as monovalent vaccine or as measles-mumps-rubella-varicella (MMRV) vaccine. In a case control efficacy study of monovalent vaccine, two doses of varicella vaccine were significantly more protective against breakthrough chickenpox than one dose (149). Anamnestic humoral and cellular immune responses to VZV after two doses of vaccine given months or years apart have been reported in several studies (3).

The observation of breakthrough varicella in about 15% of children following one dose of vaccine prompted analyses of whether this phenomenon could be due to primary or secondary immune failure. A result of either or both could be an accumulation of young adults who are susceptible to varicella a decade or so after vaccination. A study of 148 children indicated that the rate of primary vaccine failure was 24%, based on FAMA testing for VZV antibodies in the first 4 months after vaccination (111). An epidemiological study of children in Antelope Valley, CA, indicated that there was an increase in breakthrough varicella with time (over a 9-year period), with increasing severity of breakthrough cases (150). These data were interpreted to represent immunity's waning with time (secondary vaccine failure). In contrast, however, a case control study did not reveal any decrease in immunity in years 2 to 8 after immunization (143). The issue of whether waning immunity occurs after receipt of one dose of vaccine is therefore controversial but seems unlikely based on recent information (151). In any case, a recommended second dose of vaccine for all children addresses the possibility of both primary and secondary vaccine failure following one dose.

Varicella vaccine has been used effectively to protect immunocompromised children. It is no longer recommended that children with leukemia be vaccinated because of the development of vaccine-associated rash in roughly 50% that often requires treatment with acyclovir (ACV) (152). In contrast, children with HIV infection whose immune function remains relatively intact (more than 15% CD4 lymphocytes) have tolerated varicella vaccine as well as healthy children have (153, 154). Two doses at least 1 month apart are recommended for HIV-infected children. Vaccination against varicella protects HIV-infected children from both varicella and zoster (155). Vaccination of children prior to renal transplantation has also been highly successful in preventing severe varicella and also zoster (3). Small studies suggest that vaccination after liver transplantation may be safely accomplished (156).

Vaccinated individuals are at risk of developing zoster, but the incidence appears to be lower after vaccination than after natural infection (Figure 12) (157). Possibly this is because after vaccination the viral latency load is lower than after natural infection. A small study of healthy adult vaccinees also suggests similar protection against zoster (158). One third of the reported zoster cases in vaccinees are caused by the wild type virus (159).

Significant progress has been made in development of a vaccine to prevent zoster in the elderly. Interest in this approach was generated by the recognition of immunologic boosting that accompanies reexposure to VZV and the loss of VZV CMI prior to developing zoster. In this case, the rationale behind prevention of zoster with vaccination is to minimize reactivation by increasing CMI to VZV in persons who have latent infection.

Zoster can be prevented by immunizing elderly individuals with VZV latent infection due to a prior attack of varicella. This was shown in a double-blind placebo-controlled study involving 38,546 healthy adults over the age of 60 years, which was conducted from 1999 to 2004 (4). The dose of vaccine used, marketed as Zostavax (Merck and Co.), contains about 14 times the dose of live virus that is present in monovalent varicella vaccine (Varivax) in the United States (not less than 19,400 PFU per 0.65-ml dose). The vaccine proved to be extremely safe; adverse events were similar in vaccinees and placebo recipients, with the exception of transient reactions such as rash or redness at the injection site in 43% of vaccinees, compared to 17% in controls. Although the development of zoster was not the original primary endpoint of this study, a decrease in incidence of zoster of 51% was found in vaccinees compared to placebo recipients. There was better protective efficacy against zoster in vaccinees aged 60 to 69 years (64%) than in vaccinees aged 70 to 79 years (41%). In the original analysis, when development of significant pain, not zoster itself, was the primary endpoint, there was a 61% reduction of significant pain in vaccinees (some vaccinees had mild zoster with only minor or no pain). Vaccination was also highly effective in prevention of PHN, especially in patients aged 70 to 79 years (55% effectiveness). Efficacy persisted for ~8 years after vaccination (160).

A subunit (glycoprotein E) well-tolerated vaccine mixed with the adjuvant $As01_B$ (called HZ/su) was recently reported to have over 97% efficacy in elderly individuals (50 to > 70 years of age) who were followed for ~3 years. In a double-blind placebo-controlled study of 15,411 subjects in 18 countries, the incidence of HZ was 0.3 in vaccinees and 9.1 in placebo recipients per 1000 person-years of observation (161). This vaccine has not yet been licensed for use, but rapid licensure seems likely.

These vaccines against zoster are important clinical advances, although many questions remain, such as the best age at which to immunize, the duration of the effectiveness of the vaccine, and whether booster doses are necessary.

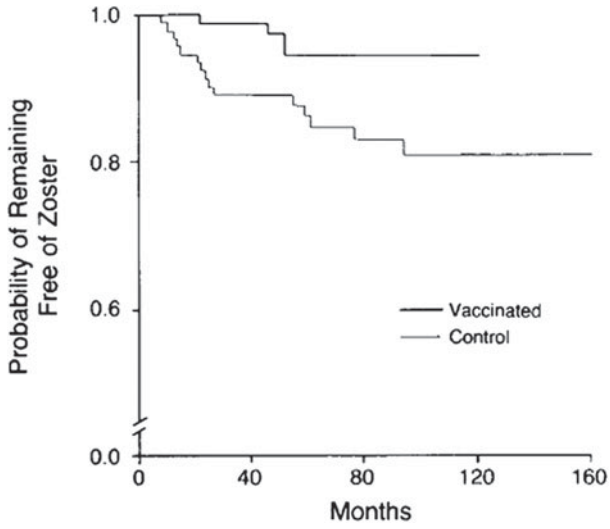

FIGURE 12 Kaplan-Meier product limit analysis of the probability of remaining free of zoster in 96 children with leukemia who had naturally acquired varicella before or after the diagnosis of leukemia. (Reprinted from reference 157 with permission of the Massachusetts Medical Society. All rights reserved.)

Drug Prophylaxis

The antiviral drug ACV has been examined for prevention of varicella in exposed persons (162). Despite apparent prevention of clinical varicella in almost all cases in such studies, the approach is not recommended in countries where passive and active immunization is available. Whether there is long-term maintenance of immunity after prophylactic ACV is not known. The CDC recommends postexposure vaccination for healthy nonpregnant varicella-susceptible individuals who have been exposed to VZV (2). Long-term ACV therapy (1 year) as a preventive against development of zoster in patients who have undergone bone marrow transplantation appears to be useful, as well as in patients who have undergone stem cell transplantation (163, 164).

Treatment

Specific antiviral therapy became available in the mid-1970s. The first drugs used were relatively ineffectual and also toxic. The modern age arrived with the introduction of ACV, which as its triphosphate is an inhibitor of DNA polymerase and a DNA chain terminator that is highly effective against VZV (165). Current recommendations for antiviral therapy for VZV are summarized below. For additional details the reader is referred to chapter 12.

ACV is extremely useful for therapy of VZV infections. Patients with severe or potentially severe infections should be treated with the intravenous formulation at dosages of 30 mg/kg/day for adults and adolescents and 1,500 mg/m^2/day for children (both given in three divided doses). Orally administered ACV is less reliable for immunocompromised patients because only about 20% of this formulation is absorbed from the gastrointestinal tract. ACV is excreted by the kidneys; patients with creatinine clearances of less than 50 ml/min/1.73 m^2 should receive one-half to one-third of this dosage. Intravenous ACV is infused over at least 1 h, with maintenance fluids given both before and during the infusion. Adequate hydration is important to prevent precipitation of the drug in the renal tubules, which can result in increases in serum creatinine levels. This complication of ACV has also been associated with bolus administration. Other adverse effects of ACV include phlebitis, rash, nausea, and neurologic manifestations such as headache and tremor. In general, however, ACV is extremely well tolerated.

Because ACV has little associated toxicity, and therapy within 2 to 3 days of onset has been associated with the best outcomes, it is recommended that early therapy be instituted for patients at high risk of developing severe VZV infections, such as children with leukemia, in order to prevent dissemination of the virus, as well as patients with ophthalmic zoster (166, 167). Therapy with ACV not only is potentially lifesaving in immunocompromised patients with VZV infections but also it prevents considerable morbidity. In zoster patients, use of intravenous ACV is associated with more rapid healing of skin lesions and resolution of acute pain than if no specific treatment is given (Table 4) (166, 167).

Orally administered ACV may be used for treatment of varicella and zoster in otherwise healthy adults and children, although most of these infections are self-limited. Dosages used are 4 grams per day (in five divided doses) for adults and 80 milligrams per kilogram per day (in four divided doses) for children. Double-blind placebo-controlled studies with healthy children and adolescents given 80 milligrams of ACV or placebo per kilogram a day for 5 days, beginning within 24 hours of onset of the varicella rash, revealed that the number of chickenpox skin lesions was reduced by ACV therapy. There was, however, only about 1 less day of fever, and children who were treated with ACV did not return to school any more rapidly than those who received placebo, reflecting a modest benefit conferred by ACV therapy. Therapy with oral ACV did not prevent complications or prevent spread of VZV (168). The therapeutic effect of oral ACV is no more pronounced in adults with varicella than in children (169). Because of the possibility of a therapeutic effect with the use of a very safe drug, however, it has become customary to treat most immunocompetent adolescents and adults who develop varicella with ACV or a related compound.

For zoster, drug efficacy has been shown even if 3 days have elapsed since onset, but the earlier ACV is begun, the

TABLE 4 Drugs for treatment of VZV infections[a]

	Dosage for treatment		
Drug	Varicella	Zoster	Comments
Acyclovir	20 mg/kg q.i.d. p.o.[b] for 5 days, 500 mg/m^2 every 8 hr i.v.	800 mg 5 times/day p.o. (adult dose) for 7–10 days, 10 mg/kg every 8 h i.v.	i.v. drug of choice for immunocompromised patients; p.o. administration shortens course of varicella by ca. 1 day in children and decreases acute pain of zoster
Valacyclovir (p.o.)	20 mg/kg t.i.d. p.o. for 5 days, age 2–18 yrs	1 g t.i.d. for 7 days (adult dosage) p.o.	High oral bioavailability; decreases ZAP duration; 0.5% of immunocompromised patients develop hemolytic-uremic-like syndrome
Famciclovir (p.o.)	NI	500 mg t.i.d. for 7 days (adult dosage)	High oral availability; decreases ZAP duration
Foscarnet	Dosage for children not established	120 mg/kg/day divided TID (adjust for renal function) i.v. up to 3 weeks	For ACV-resistant VZV infection i.v.

[a]q.i.d., four times a day; t.i.d., three times a day; p.o., orally; i.v., intravenously; NI, not indicated because of lack of supporting data; ZAP, zoster-associated pain (includes PHN).
[b]Maximum per day, 800 mg.

greater the therapeutic effect. There is some indication that early ACV therapy may decrease acute pain associated with zoster (170). Therefore, it is recommended that elderly patients with early zoster be given prompt antiviral therapy because the older the patient with this disease, the more likely it is that pain will be a future problem. One study showed no difference in outcome in older patients with zoster whether they were treated with ACV for 7 or 21 days (171). In this study, recovery was no more rapid, nor was PHN less likely to occur, if a 3-week course of tapering prednisolone (beginning at milligrams per day) were given along with ACV.

Famciclovir (FCV), is a prodrug of the antiviral penciclovir, to which it is rapidly converted in the body after oral administration. Penciclovir has an antiviral action similar to that of ACV. FCV was approved by the FDA in 1994 for oral therapy of zoster in adults. An advantage of FCV over ACV is that FCV is given three times a day (500 milligrams per dose) rather than five times a day, which may lead to better patient compliance. One study suggests that FCV given to elderly patients with zoster early in the course of infection decreases the duration of PHN but not its incidence (172). There are no data as to whether varicella may be successfully treated with FCV, nor are there any published data on use of FCV in immunocompromised patients or in children.

The prodrug of ACV, valacyclovir, which is also given orally, reaches levels in blood that are about four times higher than ACV levels after oral administration. Valacyclovir is approved for use in the United States, and one study suggested that it may be superior to ACV in immunocompetent patients with zoster (173). Valacyclovir may not be as effective as intravenous ACV, however, in highly immunocompromised adults, particularly those with AIDS with severe VZV infections (174). The dosage of valacyclovir for immunocompetent adolescents and adults with zoster is 1 gram three times a day, orally. As for all antivirals, early therapy (within 3 days of onset of rash) is required for effective results.

Foscarnet is useful for treating VZV infections caused by viruses that are resistant to ACV and FCV and was approved by the FDA for this purpose. Foscarnet acts by directly inhibiting the DNA polymerase of VZV (175). Intravenous foscarnet has been used for adults at a dosage of 120 milligrams per kilogram per day (in three divided doses) for VZV infections that are resistant to ACV. The dosage for children is not established. Its main toxicity is renal.

VZV retinitis is a potentially very serious but fortunately rare form of infection. Some forms require treatment with combination antiviral therapy including foscarnet and ganciclovir; consultation with an experienced ophthalmologist is strongly recommended for these patients.

Of concern about the potential widespread use of ACV is that drug resistance may develop and that resistant VZV may spread to others. At present, resistance is less a problem with VZV than with HSV. As for HSV, however, VZV resistant to ACV has been reported, most commonly for patients with underlying AIDS (176, 177). Rarely, the Oka vaccine strain of VZV has developed resistance to ACV (142, 178, 179).

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Cytomegalovirus

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Human cytomegalovirus (HCMV) was first isolated in the mid-1950s, when the new technology of cell culture became available. It was isolated independently by three different investigators and named because of its cytopathic effect (CPE), which produced large, swollen, refractile cells causing "cytomegaly." The virus is ubiquitous, having infected most individuals by early adulthood in developing countries and by late adulthood in developed countries. Most individuals show no symptoms as a result of primary infection, reactivation, or reinfection, indicating that the virus is well adapted to its normal host, which commits substantial immune resources to controlling HCMV. However, in individuals whose immune system is either immature (as in the fetus) or compromised by immunosuppressive therapy or human immunodeficiency virus (HIV) infection, HCMV can cause serious end-organ disease (EOD). Furthermore, accumulation of HCMV-specific T cells over decades contributes to immunosenescence. Thus, HCMV acts as an opportunist, damaging the very young and the very old as well as adults and children whose immune systems are impaired.

The pathogenesis of HCMV disease is complex, involving contributions from the host as well as from the virus. Increasing knowledge about the genetic composition of the virus can help to illuminate this complex series of relationships and provide a rational basis for therapeutic intervention and prevention of disease.

VIROLOGY

Classification

HCMV is a member of the *Betaherpesvirinae* subfamily of the *Herpesviridae*. This classification was originally based on its slow growth *in vitro* and strict species specificity but is now based on genetic sequence homologies among the *Alpha-*, *Beta-*, and *Gammaherpesvirinae* subfamilies. Based on restriction enzyme analysis of virion DNA, there are multiple genetic variants, termed strains. These genetic differences do not allow classification into distinct genotypes.

Likewise, the corresponding differences in antigenic constitution do not allow distinct serotypes to be defined. Strains are still best characterized as having an antigenic mosaic, which is recognized broadly by the host cellular and humoral immune responses. Individuals infected with one strain of HCMV thus have cross-reactive immunity against all strains, although the extent to which this provides cross-protection from disease remains to be defined.

The International HCMV Workshop in 1993 agreed upon a nomenclature for the description of HCMV proteins, which is used in this chapter (1). The system designates p for protein, gp for glycoprotein, or pp for phosphoprotein, followed by the genetic locus and any preferred trivial name in parentheses. For example, gpUL55 (gB) is the glycoprotein encoded by the 55th open reading frame (ORF) in the unique long (UL) region, known as glycoprotein B (gB).

Composition

Purified HCMV virions contain about 60 virus-encoded proteins and more than 70 host proteins (1). In common with all herpesviruses, the HCMV virion has three basic structural units.

Capsid

The HCMV capsid has a diameter of 125 nm and, by cryoelectron microscopic (cryo-EM) reconstruction, is made up of 162 capsomeres arranged as an icosahedral lattice with a triangulation number of 16. There are 150 hexons, which comprise 6 copies of the major capsid protein (MCP, encoded by the UL86 gene), 11 pentons (5 copies of the MCP), and, by analogy with herpes simplex virus (HSV), 1 penton that is structurally distinct and acts as the portal for DNA packaging. In HSV, the portal is a dodecamer of the UL6 protein; the HCMV homolog of this is UL104. The capsid provides the architecture to contain the viral genome, a double-stranded DNA molecule.

Tegument

Historically, the tegument has been described as an amorphous layer. Cryo-EM reconstructions of HSV have revealed a more structured protein layer (2), and it seems likely that the HCMV tegument has a broadly similar appearance. Structurally, the tegument is a link between capsid and envelope and may play a pivotal role in particle assembly. Functionally, the tegument contains key regulatory proteins, pp65 and pp71, that are delivered in significant amounts to the host cell.

pp65

Protein pp65 (ppUL83), the most abundant virion protein, accounts for about 15% of the total. It is the antigen that is detected in the diagnostic antigenemia assay and is also the

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major component of dense bodies (accounting for an excess of 90% of total virion proteins). The latter are noninfectious particles comprising a core of tegument proteins enclosed in a lipid envelope, which are formed during HCMV infection. Protein pp65 is trafficked to the nucleus very early in the infection via an unusual bipartite nuclear localization signal. The pp65 is a multifunctional protein that is required for various immunomodulatory roles: affecting presentation of immediate-early (IE) peptides (3), down-regulation of human leukocyte antigen-antigen D related (HLA-DR) (4), inhibiting natural killer (NK) cell responses (5), and antagonizing aspects of interferon response (6) known to be activated during viral infection (7). The protein also binds another tegument component, the viral protein kinase UL97 (8). Immunologically, pp65 is a significant target for CD4 and CD8 T-cell responses (9). Remarkably, a virus mutant lacking pp65 has no growth defect *in vitro*–except that no dense bodies are formed (10)–which would be consistent with the premise that pp65 is required for the evasion of host immune responses *in vivo*.

pp71

Protein pp71 (ppUL82) is rapidly translocated to the nucleus after virus entry. It is a transcriptional transactivator, recognizing promoters with AP1 or activating transcription factor (ATF) sites in their regulatory regions, particularly the major IE promoter (MIEP) (11). This transactivation is enhanced by an interaction with another tegument protein, ppUL35. As a potent transactivator, pp71 is analogous to the VP16 virion transactivator protein of HSV. In addition, pp71 overcomes hDaxx-mediated suppression of HCMV replication and is thus essential for the onset of productive replication (12). Another key binding partner of pp71 is the retinoblastoma protein (13), an interaction that provides sensitive regulation of a key cellular protein that is also required for efficient viral replication (14).

pp150

The basic phosphoprotein ppUL32 (pp150) accumulates late in infection at the putative sites of virus assembly, and a UL32-negative virus fails to complete the cytoplasmic stages of egress, consistent with a role in envelopment (15). The tight physical association of pp150 with capsids (16) emphasizes the presumed structural role of tegument in linking capsid with envelope and perhaps in driving virus assembly. Protein pp150 is an immunodominant B-cell target.

Envelope

The viral envelope is a host-derived lipid bilayer that contains viral glycoproteins. Envelopment is thought to occur in the endoplasmic reticulum (ER)-Golgi intermediate compartment (17), and the envelope is thus most likely derived from membranes in this region of the cell (see below). The HCMV genome codes for over 50 predicted glycoproteins, 19 of which are detected in mass spectrometric analysis of purified virions (18). The envelope glycoproteins represent the main point of contact between intact virus and host, and as such they are important inducers of neutralizing antibodies.

An important subset of envelope glycoproteins mediates virus entry (Table 1). The entry glycoproteins fall into two groups: those that are conserved throughout herpesviruses, i.e., gpUL55 (gB), gH, gL, gM, and gN, and those limited to Betaherpesviruses, i.e., gO, gUL128, gpUL130, and gpUL131. The precise role of these proteins remains debated although the conserved glycoproteins appear important for gB-dependent fusion in all cell types whereas the pentameric complex (including UL128/130/131) may act through a distinct mechanism (19) that is an important determinant of tropism for endothelial/epithelial cells (20).

TABLE 1 HCMV envelope glycoproteins

Complex in envelope	Constituent protein(s)	Mapped ORF
gCI	gB	gpUL55
gCII	gM	gpUL100
	gN	gpUL73
gCIII	gH	gpUL75
	and	
	gL	gpUL115
	plus	
	gO	gpUL74
	or	
	gH/gL	
	plus	gpUL128
		gpUL130
		gpUL131A

The HCMV envelope glycoproteins appear to exist in three distinct complexes: gCI, gCII, and gCIII (21). The gCI is made up of oligomers of gB, which are thought to have a role in attachment, fusion, and receptor binding (see below). The gB monomer (Fig. 1) comprises two subunits, gp116 and gp55, derived by furin cleavage of a precursor. This cleavage is not essential for virus infectivity *in vitro* (22). The protein subsequently undergoes further processing and oligomerization (23). Studies of the crystal structure of HSV gB have shown a homotrimer, and HCMV gB probably shares this overall structure (24).

The gCII comprises heterodimers of gM (UL100) and gN (UL73). This complex plays a role in virus attachment via the host cell proteoglycan-binding activity of gN. Both components also have roles in assembly (25).

The gCIII is based on the disulfide-linked heterodimer of gH (UL75) and gL (UL115). This in turn interacts with gO (UL74) or with gpUL128, gpUL130, and gpUL131A, forming two or more distinct complexes (26). Virus-free fusion assays have implicated gH/gL alone as a fusogenic complex (27).

A number of other envelope glycoproteins have been recognized. gpUL4 is a glycoprotein of unknown function that is expressed with early kinetics. HCMV encodes four chemokine receptor homologs, US27, US28, UL33, and UL78. Two (UL33 and US27) are known to be in the virion (28). In contrast to most host receptors, the viral CCR homologs are constitutively activated. Those in the virion are theoretically able to signal as soon as they become incorporated into host membranes following virus entry. US28 induces migration of smooth muscle cells (29), and this is perhaps significant in terms of an association of HCMV with vascular disease.

Genome

The most accurate representation of the HCMV genome has come from comparisons of the complete genomic sequence of a minimally passaged strain, Merlin, with partial sequences from other strains, including some obtained directly from clinical material (30). HCMV genomic analyses have

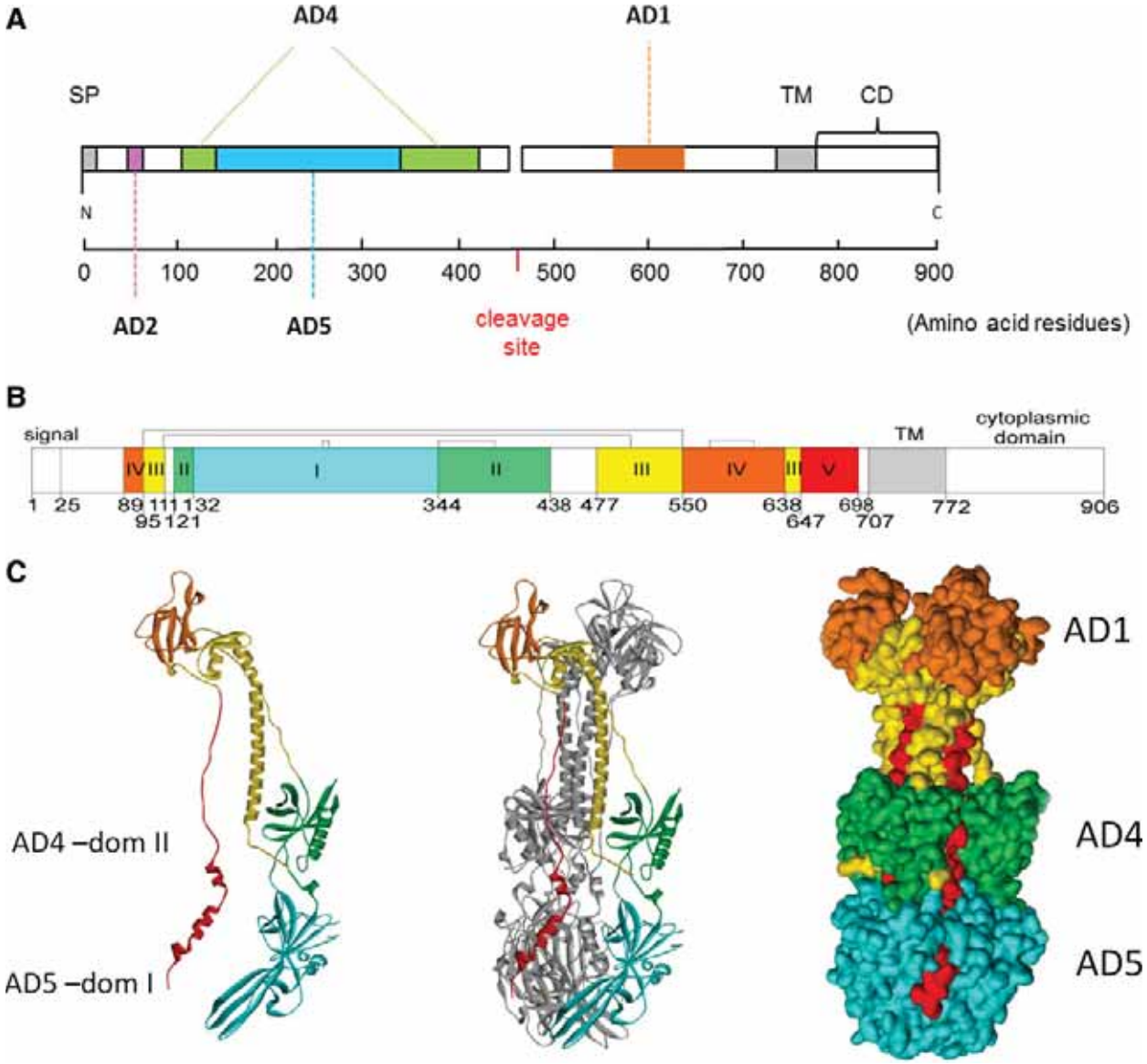

FIGURE 1 Schematic representation of glycoprotein B from HCMV. A: The locations of the known antigenic domains (AD) mapped onto gB. B: Linear representation of domain architecture. C: gB monomer, trimer, and three-dimensional representation showing domains. AD1 is a linear, immunodominant epitope, AD2 a linear epitope, AD3 a linear epitope located where the transmembrane portion of gB abuts the cytoplasmic portion, AD4 a discontinuous epitope, and AD5 a discontinuous epitope. SP, signal peptide; TM, transmembrane domain; CD, cytoplasmic domain; dom, domain. Adapted from Potzch *et al.*, (2011) *PLOS Pathogens* vol 7, issue 8; e1002172.

been complicated by the tendency of the virus to lose a 22-kilobase (kb) segment of the genome, termed ULb′, rapidly upon isolation and passage in fibroblasts (31). The ULb′ segment contains genes that are not essential for replication *in vitro* but ones that play key roles in immune evasion and in determining cell tropism.

The HCMV Merlin genome is a 235,645-base pair (bp) linear double-stranded DNA molecule with a coding capacity of 165 genes (30). It is the largest genome among the human herpesviruses. The genome comprises two unique regions, termed UL and unique short (US), flanked by repeated sequences. Inversions of the unique regions give rise to four isomeric forms of virion DNA present in equimolar amounts. No pathological significance has been attributed to these isomers.

The Merlin genome (Fig. 2) has a central section that contains the core genes, approximately 40 genes that are conserved throughout the *Alpha-*, *Beta-*, and *Gamma-herpesvirinae*. Many of the other genes are grouped into families of related genes (Fig. 2) thought to have evolved by duplication events. There are numerous spliced genes. MicroRNAs have been detected across the genome encoded by both strands (32–34). A functional RNA is encoded that regulates mitochondrial-induced cell death (35). The UL36-38 gene region encodes potent inhibitors of apoptosis which inhibit activation of caspase 8 (UL36), mitochondrial activation (UL37) exon 1 and apoptosis following endoplasmic reticulum stress (UL38; UL37).

Sequence variation occurs across the genome but appears to be highest at either end of the UL segment. A number of

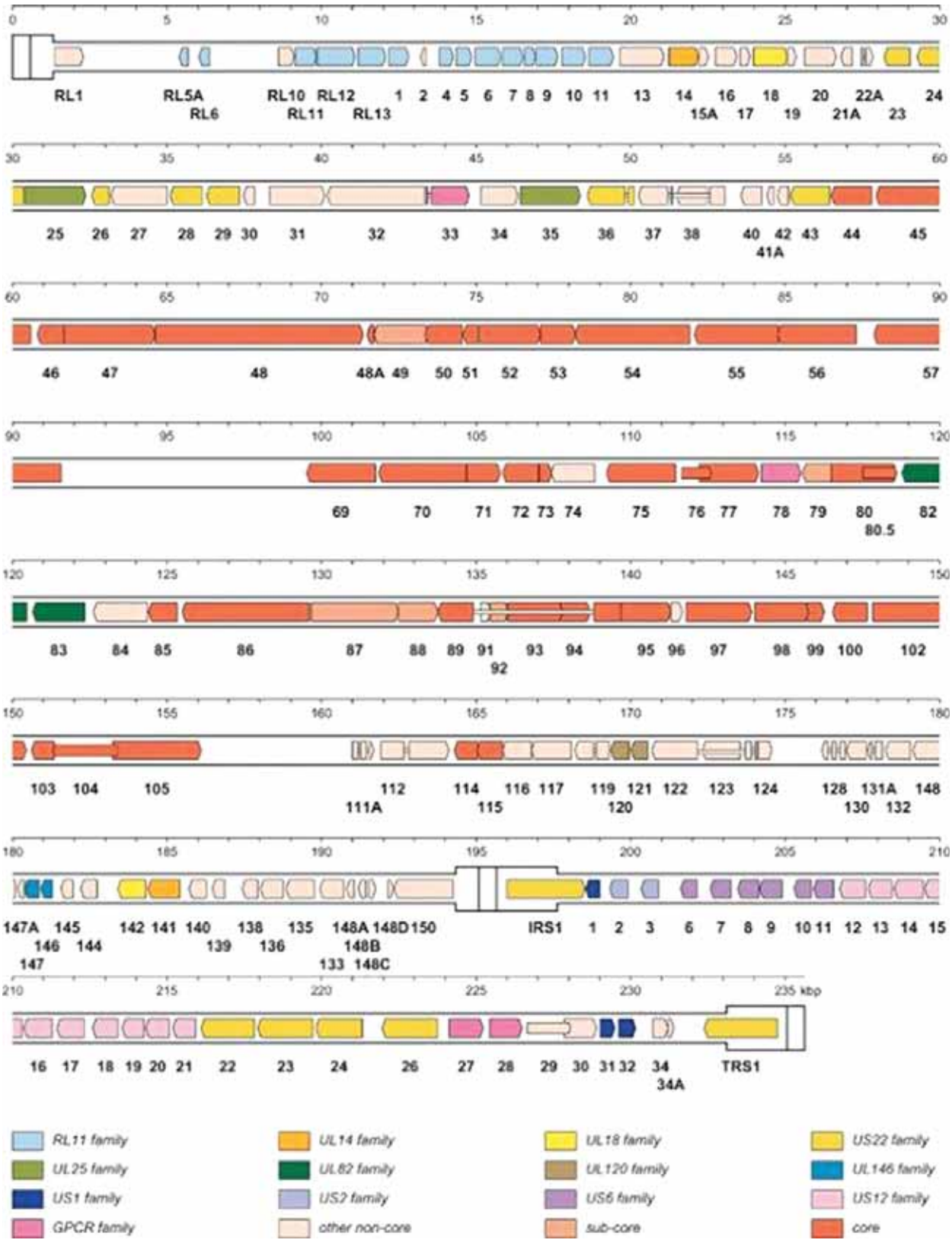

FIGURE 2 Consensus genetic map of wild-type HCMV based on the Merlin genome. RL and RS (which contain the *a* sequence as a direct repeat at the genome termini and as an inverted repeat internally) are shown in a thicker format than UL and US. Protein coding regions are indicated by colored arrows grouped according to the key, with gene nomenclature shown below. Introns are shown as narrow white bars. Genes corresponding to those in AD169 RL and RS are given their full nomenclature, but the UL and US prefixes have been omitted from UL1–UL150 (12 to 194 kbp) and US1–US34A (199 to 231 kbp). Colors differentiate between genes on the basis of conservation across the *Alpha-*, *Beta-*, and *Gammaherpesvirinae* (core genes) or between the *Beta-* and *Gammaherpesvirinae* (sub core genes), with subsets of the remaining noncore genes grouped into gene families. GPCR, G protein coupled receptor. (Reprinted from reference 30 with permission).

genes, notably UL146, which encodes a functional alpha chemokine, as well as UL73, UL74, and UL144, show a remarkable level of sequence variation (36). The functional significance of this variation is not known. "Next generation" sequencing techniques revealed extensive genetic diversity on an order of magnitude greater than previously identified (37). It remains possible that these techniques accurately document genetic variation in transcription but that most changes are deleterious to infectivity so that the CMV virion produces a genetic bottleneck containing a relatively conserved genome. Ribosomal profiling revealed that genetic information was being translated from regions of the genome that were not recognized as distinct ORFs (38). Indeed, proteins may be produced from 700 distinct regions of the genome rather than from the 165 named ORFs, although confirmation of these findings is needed.

Replication Cycle

Virus Entry

Like other herpesviruses, primary attachment of HCMV is mediated by interactions between virion glycoproteins and sulfated proteoglycans on the host cell surface (39). This primary attachment serves to bring the virus into close association with the target cell to allow receptor binding. HCMV entry can occur by direct fusion between the virion envelope and the plasma membrane or via endocytosis followed by fusion from within the endosome (40, 41). The cellular and viral factors contributing to the choice of pathway are not known. Three glycoproteins—gB, gH, and gL—constitute the core herpesvirus entry machinery. The roles played by each protein in the entry process remain unknown.

The crystal structure of HSV gB shows convincing similarities to the G protein of vesicular stomatitis virus, a known fusion protein (42). HCMV gB appears to be fusogenic when stably expressed in U373 cells, although fusion assays have shown that HCMV gH/gL alone can mediate fusion (27), and HSV gH/gL has certain characteristics that resemble fusion proteins. These proteins cooperate closely to mediate fusion, and coimmunoprecipitation studies show that HCMV gB and gH interact transiently during virus entry (43).

The gH/gL heterodimer is found in at least two distinct complexes (Table 1), one with gO and one with glycoproteins encoded by members of the genes from UL128 to UL131. The latter have been shown to have a role in mediating entry to endothelial cell types, but not fibroblasts (44), and blocking studies with soluble UL128 protein suggest that this may bind a receptor on endothelial cells (43). The use of different forms of gH/gL complex as a means of regulating host cell tropism is an emerging theme in the Beta- and Gammaherpesviruses (45), although recent data challenge this view (19) and instead argue that gH/gL/gO is required for gB-mediated fusion in all cell types and that the pentameric complex defines endothelial cell tropism through an additional undefined mechanism.

The virus cell contacts that are a precursor to entry induce a range of signaling events, but their role in the context of virus entry is not clear. Various candidate receptors for gB and gH/gL have been proposed, but there is as yet no consensus as to their role in entry.

Transport to the Nucleus

After entry, the tegument and capsid are released into the cytoplasm. Transport of the capsid to the nucleus occurs in a microtubule-dependent manner, and capsids appear to retain some tegument throughout this process (46). The viral genome is released from capsids when they reach the nuclear pores. The mechanism underlying localization and release of the genome is unknown, although recent data implicate the UL47 tegument protein in this process (47).

Transcription and Gene Expression

HCMV gene expression, in common with other herpesviruses, occurs in three phases, termed immediate early (IE), early (E), and late (L). In general, IE genes encode regulatory proteins, E genes encode replication proteins, and L genes encode structural proteins. The IE genes are the first to be transcribed in the replication cycle. A defining characteristic is that they are transcribed in the absence of viral protein synthesis. There are five IE transcription loci in the genome: UL36-37, UL122/123, UL119-115, US3, and TRS1. The most abundant, and by far the best studied, transcripts are those arising from the major IE locus: UL122/123. Expression of these genes is controlled by the MIEP, well known from its widespread use in expression plasmids. The MIEP is complex, with multiple transcription factor binding sites in the upstream enhancer element. This combination of sites may well play a role in allowing IE gene expression to occur in a diverse array of cell types, perhaps containing a varied repertoire of transcription factors. Broadly speaking, the MIEP is active in differentiated cell types and is repressed in undifferentiated cell types. This differential activity appears to be a significant regulator of latency and reactivation.

Though numerous gene products are produced through intricate patterns of splicing, the two major products are the IE1 and IE2 proteins (Fig. 3). These two proteins have a common N-terminal region, corresponding to exons 2 and 3 of the gene locus; they differ in the C-terminal region, IE1 corresponding to exon 4 and IE2 corresponding to exon 5.

MIEP-driven transcription is favored by events that occur concurrently with virus entry. Cell surface binding by gB and gH induces NF-*k*B and Sp1 expression (51), which could favor MIEP activation due to the presence of binding sites for both these transcription factors. A more important aspect is the delivery of the virion transactivator, pp71, which is released as soon as incoming virions are uncoated (11) and targets a number of antiviral host factors to drive MIE viral gene expression. The detailed functions of the IE1 and IE2

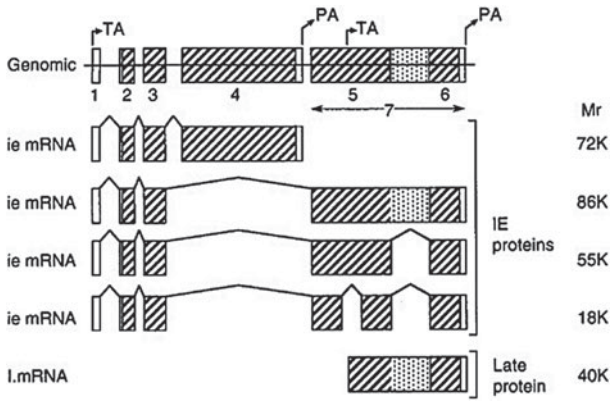

FIGURE 3 The major IE region of HCMV showing the splicing events that produce distinct proteins. TA, transcriptional activation; PA, polyadenylation; l.mRNA, late mRNA.

proteins have been reviewed (52). IE1 binds PML and disrupts ND10 (53). An IE1-negative virus fails to replicate at a low multiplicity of infection. This deficit can be overcome by histone deacetylase (HDAC) inhibitors (54), highlighting the importance of these molecules in the regulation of MIEP activity (see below). Binding of IE1 to HDAC3 promotes transcription by antagonizing histone deacetylation through sequestration away from target viral promoters. This drives acetylation of histones bound to viral promoters driving viral gene expression (54). IE1 is also capable of direct transactivation (55).

IE2 is essential for virus replication. It has dual roles in regulating expression of viral and cellular genes and negatively autoregulating the MIEP via an upstream *cis*-acting repressive sequence (Fig. 4). IE2 also binds HDAC3, potentially resulting in an effect similar to that of IE1 (54) although this could also contribute to repression of MIEP seen at late times of infection (56). IE2 localizes to ND10-like structures in association with the viral genome. These foci develop into replication compartments, consistent with a role for IE2 in the early stages of genome replication (57). Indeed, IE2 binds a promoter in the lytic origin of replication in complex with the E protein UL84 (see below).

Both IE1 and IE2 activate E and L gene expression. E genes are defined as those genes that (i) require prior expression of the IE genes for transcription and (ii) can be expressed in the presence of inhibitors of DNA replication, such as ganciclovir and foscarnet.

DNA Replication, Capsid Assembly, and DNA Packaging

In contrast to transcription, for which the virus relies on cellular machinery, the virus encodes its own DNA replication apparatus. Thus, viral DNA replication is a key target for the development of antiviral drugs. HCMV also stimulates progression of the host cell cycle toward S phase yet suppresses host DNA synthesis, thus ensuring a cellular environment suitable for viral DNA replication (58). Upon delivery to the nucleus, genomes are deposited at ND10 sites, where transcription and DNA replication both occur (59).

The input genome is thought to be circularized independently of viral gene expression. Circularization is consistent with a rolling-circle mode of replication, but this has not been proven for HCMV. Indeed, HCMV genome replication intermediates are more complex than would be produced by this method (60).

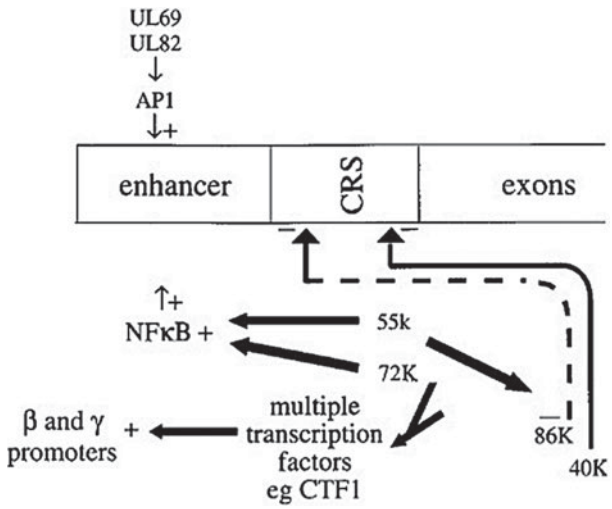

FIGURE 4 Regulation of the major IE region of HCMV. CRS, *cis* repression sequence.

TABLE 2 Eleven loci required for HCMV replication

Protein(s)	Locus
DNA polymerase	UL54
Polymerase-associated protein	UL44
ssDNA[a] binding protein	UL57
Helicase-primase	UL70
	UL105
	UL101–102
Transactivators	UL36–38
	IRS1 (or TRS1)
	IE1/2
Unknown functions	UL84
	UL112–113

[a]ssDNA, single-stranded DNA.

Eleven loci (Table 2) are required for HCMV genome replication (61). The core replication proteins corresponding to UL54 (DNA polymerase), UL44 (DNA polymerase accessory protein), UL57 (single-stranded DNA binding protein), UL105 (helicase), UL70 (primase), and UL102 (primase-associated factor), which are present in all herpesviruses, provide the basic DNA replication machinery. However, the details of their function and interactions are unknown (62).

The origin of replication, *ori*Lyt, is adjacent to the UL57 gene, essential for virus replication, and distinct from other viral origins (63). It is structurally complex and contains numerous short repeated sequences, including transcription factor binding sites, and has an overall asymmetry of nucleotide distribution, being AT rich on the left side but GC rich on the other (64). Transcripts are produced within *ori*Lyt (65), and two viral RNAs are present as RNA/DNA hybrids within the region in packaged genomes (66). Given the absence of an obvious origin binding protein in HCMV, it seems likely that these are in some way involved in initiation of replication (66); indeed, the UL84 protein, which is required for genome replication, binds stably to this region of *ori*Lyt (62). The initiation of L gene expression, which is, presumably, in some way regulated by the onset of DNA replication, is not understood.

Capsid Assembly

Based on their structural similarities, assembly of HCMV capsids is likely to resemble that of HSV capsids. The MCPs are conserved throughout the herpesviruses.

A transient internal scaffold coordinates assembly of the nascent capsid (67). This scaffold contains two major components encoded by the UL80 gene region: the protease precursor (UL80) and the assembly protein, pAP (UL80.5), which comprises the C-terminal half of the protease. The first steps in capsid assembly occur in the cytoplasm, where pAP interacts with itself and with the MCP and causes the translocation of these capsid protomers to the nucleus (68). Once in the nucleus, pAP associates with the protease precursor and other capsid components are recruited to the protomers, giving rise to procapsids. Autoproteolysis of the protease then liberates the scaffold components from the nascent capsid (69).

Capsid maturation is coupled to DNA packaging. Based on data from HSV, DNA is packaged through a unique vertex containing the portal protein (UL6 in HSV). The HCMV portal homolog, UL104, forms high-molecular-weight complexes and binds DNA in a sequence-independent manner (70). HSV UL6 is thought to act as a nucleation factor for capsid assembly, explaining its asymmetric localization in the capsid.

DNA packaging requires *pac* signals located at the genome termini. These are recognized by the viral terminase complex, made up of UL56 and UL89 (71), which likely interacts with the portal protein.

Egress

The broadly accepted model for HCMV egress is the envelopment–de-envelopment–re-envelopment model. Mature capsids bud through the inner nuclear membrane (INM), acquiring a primary envelope. The resulting perinuclear enveloped virions then fuse with the outer nuclear membrane, liberating capsids to the cytoplasm. These traffic to the site of envelopment, where they acquire tegument and the final envelope. Mature virions are released from the cell by exocytosis or lysis. However, much of this pathway is inferred from studies of other herpesviruses, and some aspects of the pathway remain controversial.

The earliest step is recruitment of capsids to the INM. For HSV, the UL31 and UL34 proteins play a role in this recruitment. The HCMV homologs of these proteins, UL53 and UL50, are thought to have a similar function, and UL53 colocalizes with lamin B at the INM, consistent with a role in nuclear egress (72). Primary envelopment likely occurs by budding into large infoldings of the INM. It is not clear whether all of the tegument proteins are present in these particles. Cryo-EM tomogram images of perinuclear enveloped HSV show that the tegument is distinct from that in mature virions, suggesting that a subset of tegument proteins may be included in these particles, although not all HCMV tegument proteins have been detected in the nucleus. The primary envelope is then lost by fusion between the primary enveloped virion and the outer nuclear membrane, giving rise to naked cytoplasmic capsids.

Based on confocal microscopic analysis, the site of final assembly has been defined as a secretory vacuolar structure that expresses Rab 3, TGN 46, and mannosidase II (49). Data also support a role for endocytic compartments in assembly and final envelopment. A fluid phase marker colocalizes with sites of virus budding, and cell surface biotinylation of infected cells leads to incorporation of biotinylated gB into virions (73).

Some tegument proteins may play roles in driving the last stages of virus assembly, including the secondary envelopment step. A pp28-negative virus has cytoplasmic accumulations of nonenveloped tegumented capsids (74); accumulation of other viral proteins in the Golgi-associated assembly region is not affected. Additionally, both gM and gN (which form a heterodimer in the virion) have roles in assembly.

During the later stages of virus assembly, an interaction between phosphorylated gB and the host trafficking protein PACS-1 is involved in recruitment of gB to the site of assembly, although blocking of PACS-1 function has a modest effect on progeny titers (75). EM studies have shown an association of HCMV glycoproteins and budding particles with cytoplasmic multivesicular bodies, and depletion of endosomal sorting protein VPS4 using small interfering RNA technology leads to enhanced release of HCMV particles and implicates the multivesicular bodies as components in viral degradation (76).

BIOLOGY

Latency and Reactivation

A defining characteristic of herpesviruses is the establishment and maintenance of a latent state from which the virus can periodically reactivate to undergo productive replication. Peripheral blood monocytes are a major site of carriage of HCMV DNA in healthy individuals (77). Monocytes arise from bone marrow resident pluripotent $CD34^+$ stem cells, and this cell population appears to be a site of HCMV latency, although it is not clear if this is the only one. $CD34^+$ precursor cells are a self-renewing population, but it is not clear whether successive generations of cells are reinfected by virus produced from infected bone marrow stromal cells or circulating infected endothelial cells or whether the HCMV genome is maintained in this population by some form of replication (78), perhaps analogous to the maintenance of the latent Epstein-Barr virus (EBV) genome in replicating B cells (79). The HCMV genome in $CD14^+$ peripheral blood mononuclear cells is in a circular plasmid form (80), but there is no conclusive evidence of a latent origin of replication or of genes homologous to those required for EBV genome maintenance (79). UL84 may be involved in the replication of latent genomes (78).

$CD34^+$ monocytic precursors spend a relatively short time in the bone marrow before differentiating and moving to the peripheral blood. The regulation and initiation of reactivation appear to be governed largely by differentiation-dependent chromatin remodeling around the MIEP. Studies of the MIEP in latently infected $CD34^+$ cells and monocytes from healthy donors show that it is associated with heterochromatin protein 1 (HP-1) and non-acetylated histones, both markers of transcriptional repression. Differentiation of these cells led to loss of HP-1 and histone acetylation, providing an environment consistent with transcriptional activation. This was correlated with recovery of infectious virus, showing that HCMV reactivation is associated directly with the chromatin remodeling that occurs with differentiation (81).

Various viral transcripts have been associated with latent infection, including those from the MIE region, the viral interleukin 10 (IL-10) gene (UL111.5A), UL138, UL144, US28, and an antisense transcript from the UL81-UL82 locus containing a 133-amino-acid ORF product. The role of these transcripts in latency, and of any proteins encoded by the transcripts, is currently unknown although recent evidence points toward vIL-10 playing a key role in immune evasion (82).

HLA Modulation

HCMV has evolved to contain a series of genes that are capable of interfering with the immune response (see Chapter 14). Most of these are proteins, but one is a microRNA, and HCMV also recruits some cellular proteins, in the form of complement control proteins, to help avoid innate immune responses (Table 3). The presence of these multiple genes presumably allows HCMV to persist in multiple sanctuary sites throughout the body.

Several proteins are responsible for down-regulating the cell surface display of mature class I complexes (Fig. 5). At IE times, pUS3 binds to HLA heavy chains in the lumen of the ER and sequesters them. At E times, pUS6 blocks TAP, the

TABLE 3 HCMV immune evasion genes

Immune defense	Viral genes or host proteins
Complement	CD46, CD55, CD59
Chemokines	UL146, UL147, UL128
Chemokine receptors	UL33, UL78, US27, US28
Interferon	UL83, UL122, UL123, TRS1/IRS1
Fc receptors	UL118/119, IRL 11
IL-10	UL111.5 (vIL-10)
CTLs	UL83, US6, US3, US2, US11
NK cells	UL18, UL40, UL16, UL141, UL142
	MicroRNA miR-UL112

transporter associated with antigen presentation. At E times also, pUS2 and pUS11 re-export mature complexes back from the ER to the cytosol, where they are degraded in the proteasome. The coordinated action of these proteins produces a dramatic down-regulation of mature class I HLA complexes, blocking the ability of cytotoxic T lymphocytes (CTLs) to recognize the virus-infected cell. However, the cell would then be susceptible to lysis by NK cells, which recognize the absence of negative signals provided by non-antigen-specific HLA molecules. Accordingly, HCMV encodes two other proteins able to mimic these negative signals: gpUL18 and gpUL40 (Fig. 5). The latter protein encodes a canonical ligand within its leader sequence for HLA-E that is identical to the HLA-Cw03 signal sequence peptide. Expression of gpUL40 in HLA-E-positive cells confers resistance to NK cell lysis via the CD94/NKG2A receptor. The generation of the gpUL40 peptide ligand does not require the TAP system, and the mature gpUL40 can upregulate expression of HLA-E.

HCMV also contains other genes that interfere with the stress response of the cell, which would normally activate NK cells to destroy the cell (Fig. 5). These include the proteins encoded by UL16, UL141, and UL142, which inhibit the action of the "UL binding proteins," CD155, and an unknown stimulatory molecule. In addition, a microRNA also blocks one of the UL binding proteins (83).

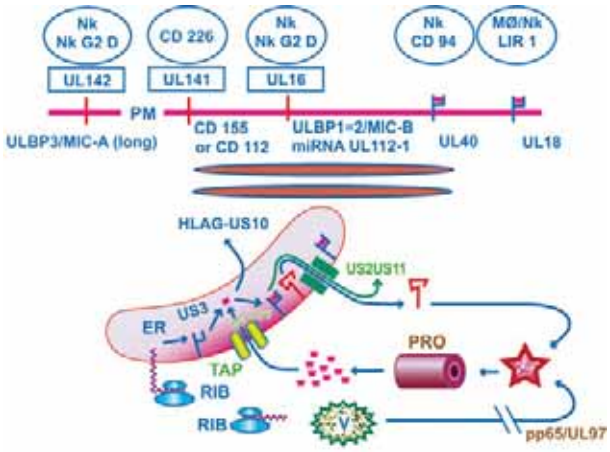

FIGURE 5 Effect of HCMV proteins on display of mature HLA complexes at the plasma membrane (PM). TAP, transporter associated with antigen presentation; RIB, ribosome; PRO, proteasome.

EPIDEMIOLOGY

Prevalence and Incidence

HCMV infection is distributed worldwide, with geographic differences explained by socioeconomic differences of exposure (50). HCMV infects both vertically and horizontally and can be transmitted by either route during primary infection, reinfection, or reactivation. Frequent shedding in saliva, semen, and cervico-vaginal secretions in the absence of symptoms enables inapparent transmission to others. Between 2 and 10% of infants are infected by the age of 12 months in all parts of the world. In later childhood, close contact facilitates transmission, so family groups, particularly those crowded together in unhygienic circumstances, have a high prevalence of infection.

In populations with upper socioeconomic circumstances, approximately 40% of adolescents are seropositive, and this figure thereafter increases by approximately 1% annually (84) (Fig. 6). Approximately 70% of adults of upper socioeconomic conditions and 90% under poorer conditions eventually become infected with HCMV.

Following primary infection, HCMV persists either in a true latent form or in a state of low-level replication made possible by the immune evasion genes described above. Thus, individual cells could exhibit HCMV latency, while particular organs could always have some cells producing virus particles. Occasional reactivations of HCMV are almost always asymptomatic but presumably allow HCMV to spread horizontally. Reactivations also transmit vertically, so congenital HCMV infection is found most frequently in the poorest communities in the world (85).

Reinfection with another, or possibly the same, strain of HCMV can also occur; comparison of virion DNA samples by restriction enzyme analysis or by sequencing can differentiate between strains. The term recurrent infection is often used when infection is non-primary but it has not been possible to differentiate reactivation from reinfection. No seasonal patterns are recognized.

Transmission

Routes of Primary Infection

Intrauterine

The greatest risk to the fetus follows primary maternal infection during pregnancy. Intrauterine infection presum-

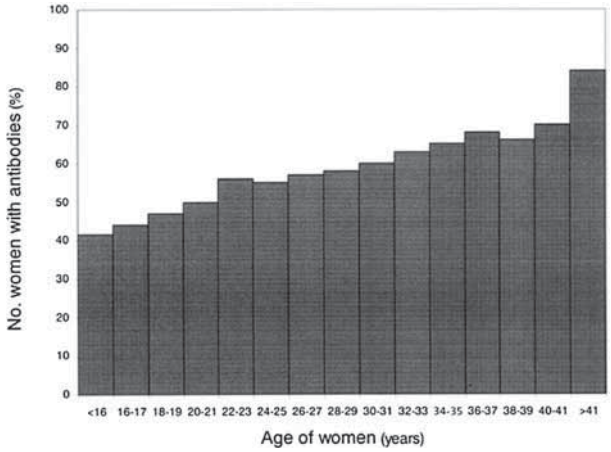

FIGURE 6 Age-specific prevalence of IgG antibodies against HCMV.

ably follows maternal viremia and associated placental infection. Intrauterine transmission of HCMV occurs in approximately 32% of pregnant women with primary infection (86). CMV-specific cell-mediated and neutralizing antibody levels would be expected to develop more rapidly in nontransmitters than transmitters, but the significance of detecting early host immune responses in pregnancy is confounded by gestational stage (87). Approximately 1% of seropositive women transmit HCMV in utero, but at present there are no laboratory markers to identify those at greatest risk. Although they have a low risk as individuals, the abundance of seropositive women in the community means that most babies with congenital CMV infection are born to women who were "immune" before conception (88).

Perinatal

Perinatal infection can be acquired from infected maternal genital secretions or breast milk. Milk is a plausible source of HCMV, because perinatal infection has occurred only when breast-feeding has taken place, not when infected women gave bottle feeds to the infants.

Postnatal

The asymptomatic nature of most postnatal HCMV infections precludes identification of the routes of transmission, but exposure to saliva or genital secretions during sexual contact is suspected, because these contain infectious virus. HCMV transmission occurs from child to child in day care centers, most commonly among young children and especially if one is known to be excreting HCMV. Transmission via fomites, such as toys contaminated by saliva, has been implicated. Infection in children is usually asymptomatic, but infectious toddlers may transmit virus to adult staff or to mothers (89–91).

HCMV seroprevalence is 90 to 100% among patients attending clinics for sexually transmitted disease and among male homosexuals. Individuals linked epidemiologically by sexual contact have been shown to excrete strains of HCMV on the cervix and in semen that are indistinguishable by restriction enzyme typing. While these findings are consistent with the sexual transmission of HCMV, formal studies have not quantified the risk of acquiring HCMV by seronegative or seropositive contacts from particular sexual practices. Individuals can be infected with more than one strain of HCMV (92).

Blood Transfusion

The introduction of extracorporeal blood perfusion in the 1960s led to a syndrome of leukopenia, pyrexia, and atypical leukocytosis, termed post-perfusion syndrome, caused by primary HCMV infection. The failure to isolate HCMV from blood from healthy donors suggests that the virus exists in a latent state, presumably within leukocytes, and is reactivated when cells encounter an allogeneic stimulus. Allogeneic stimulation of macrophages containing latent HCMV reactivates virus, which can subsequently infect and replicate in fibroblasts (93).

Although HCMV can be transmitted by blood transfusion, this is an uncommon event, occurring in only 1 to 5% of seronegative recipients exposed to seropositive blood. The risk is reduced to zero where filters are used routinely to remove leukocytes. No laboratory tests have been shown to identify donors at high risk of transmitting HCMV.

Organ Transplantation

Seronegative patients undergoing solid-organ transplantation are at no risk of primary infection from seronegative donors, whereas a seropositive organ transmits the virus in 60 to 80% of donations. Both kidneys from a single donor are usually concordant for transmission; however, it is not clear whether parenchymal cells of the organ or infiltrating leukocytes are the source of the infectious virus. Reinfection also occurs with transmission from a seropositive donor to a seropositive recipient. In contrast, typing of strains by restriction enzyme analysis of DNA demonstrates that the virus causing disease after bone marrow transplantation is derived from the seropositive recipient (94). Indeed, seropositive donors may be chosen preferentially for seropositive recipients because they can adoptively transfer some immunity into the recipient (95).

Sperm Donation

Organizations that recruit and organize sperm donors often reject CMV seropositive individuals. They collect sperm from CMV seronegative individuals and store it frozen. Three months later the same donor produces sperm and serum; if the latter shows him to still be CMV seronegative, then the earlier frozen sample is retrieved and used clinically. This system has the potential to keep CMV infection at a low rate, but has the disadvantage of rejecting the majority of men volunteering to be donors.

PATHOGENESIS

HCMV replication occurs widely in multiple tissues, as illustrated by isolation from autopsy material from AIDS patients (Table 4). During life, biopsy samples of lung, liver, esophagus, colon, and kidney are frequently found to contain HCMV. Replication has been demonstrated in polymorphonuclear leukocytes, monocytes, T lymphocytes ($CD4^+$ and $CD8^+$), and B lymphocytes (96). Thus, HCMV is a systemic infection. As described above, this ability to replicate in multiple tissues is consistent with the presence of many isoforms of transactivating proteins potentially able to function in many cell types. However, it is not consistent with the restricted range of cell types that can be infected *in vitro*. The latter finding is probably an artifact resulting from the inability to propagate and maintain in the

TABLE 4 Site of HCMV detection by cell culture in 47 AIDS patient autopsies

Site	No. (%) of cases
Esophagus	3 (6)
Stomach	3 (6)
Small bowel	5 (11)
Large bowel	7 (15)
Liver	6 (13)
Pancreas	3 (6)
Spleen	2 (4)
Trachea	4 (9)
Lung	19 (40)
Heart	5 (11)
Adrenal	17 (36)
Thyroid	4 (9)
Salivary gland	6 (13)
Brain	8 (17)
Spinal cord	1 (2)
Ganglion	2 (4)

laboratory the fully differentiated cells preferred by HCMV. An incubation period of 4 to 8 weeks can be estimated from four distinct informative clinical settings. They are perinatal infection, transmission by organ allograft, transmission by blood transfusion, and intrauterine transmission following primary infection in the mother.

Factors in Disease Production

Primary Infection

Immunocompetent infants, children, and adults usually get no symptoms from primary infection. Infection in an immunologically unprimed individual represents the greatest risk of disease in the fetuses of pregnant women (97) and recipients of solid-organ transplants (98), but this effect is not absolute. Some patients with primary infection do not have disease, whereas some with reinfection get disease. HCMV reinfection of seropositive renal transplant recipients represents more of a risk for disease than reactivation of latent virus (98). In contrast to the case with solid-organ recipients, reactivation represents the major source of HCMV disease in bone marrow transplant patients (94). Likewise, HIV-positive patients rarely experience primary HCMV infection; their disease results from either reactivation of latent infection or reinfection.

Viremia

Imbalance between the rate of production of HCMV at sites of infection and its effective rate of immune clearance may produce viremia. Subsequently, viremia seeds target organs, which may or may not have sufficient local defenses to prevent disease. The quantity of HCMV required to cause disease is predicted to vary considerably, depending on the precise details of the pathogenic mechanisms. Thus, immunopathology may be triggered at low viral loads but remain present at high loads. Moderate loads may cause disease through cell lysis secondary to viral replication. Finally, very high viral loads might damage target cells when HCMV binds to them, a process we term direct toxicity. This hypothetical scenario will become increasingly susceptible to investigation using measures of viral dynamics. Because HCMV DNA can also be detected in the plasma of patients (99), it will be important to determine if plasma viremia marks a distinct phase of pathogenesis.

In recipients of solid-organ transplants or bone marrow, the detection of active infection in saliva or urine using conventional cell culture or PCR has a relative risk of approximately 2 for future HCMV disease (100). In contrast, the detection of HCMV in the blood has a relative risk of 5 to 7, irrespective of methods of detection employed (100). This provides the scientific basis for administering antiviral drugs to patients with active HCMV infection before they develop symptoms. For patients with viremia, the term preemptive therapy is used, whereas the term suppression has been used for those with viruria. Viremia is not an absolute marker of disease but has a strong positive predictive value (Table 5). Presumably, viremia indicates that innate immune responses at local sites of infection have been inadequate. Viremia allows HCMV to seed multiple organs, but some transplant patients can presumably mount adequate regional innate or adaptive responses to prevent HCMV disease. This high risk for HCMV disease indicates that preemptive therapy should be considered in patients with viremia (101). Note that in the special case of HCMV pneumonitis in transplant patients, especially bone marrow transplant patients, the HCMV immune response may contribute to disease (i.e., HCMV pneumonitis may be an immunopathological condition that, once initiated, does not respond well to effective anti-HCMV therapy) (102). Nevertheless, the incidence of such disease can be reduced significantly if antiviral therapy is given as suppression (103), preemptively (104), or as prophylaxis (105). This emphasizes the importance of targeting of antiviral drugs at the earliest possible stage of infection, rather than when the patient has disease.

TABLE 5 Comparison of PCR and culture for 150 immunocompromised patients

	Value in blood[a]				
Assay	Sensitivity	Specificity	PPV	NPV	RR
Culture	0.26	0.93	0.5	0.82	3.4
PCR	0.8	0.86	0.62	0.94	5.84

[a]PPV, positive predictive value; NPV, negative predictive value; RR, relative risk.

Virus Replication

HCMV replication patterns range widely depending on the type of infection and host population. In 1975, Stagno and colleagues (90) showed that among neonates with congenital HCMV, the titer of viruria was significantly increased among those with disease or those destined to develop disease (Fig. 7). The kidney is not a target organ for HCMV disease in neonates, so the quantity of viruria presumably reflects systemic infection in which viral production in the kidney acts as a marker for replication in the target organs, which are less clinically accessible (brain and inner ear) (106). In the case of the neonate, this observation is even more remarkable, given that the urine is sampled after birth, yet infection may have occurred months earlier in utero (97). Nevertheless, by 3 months of age, the significant difference between viral loads in the two groups of neonates is no longer present (90), presumably reflecting the ability of the neonate's immune response to control active replication.

The advent of PCR enabled greater sensitivity of detection of HCMV to be achieved, and quantification by real-time PCR can be used to determine HCMV load and thereby gain insight into HCMV pathogenesis in transplant

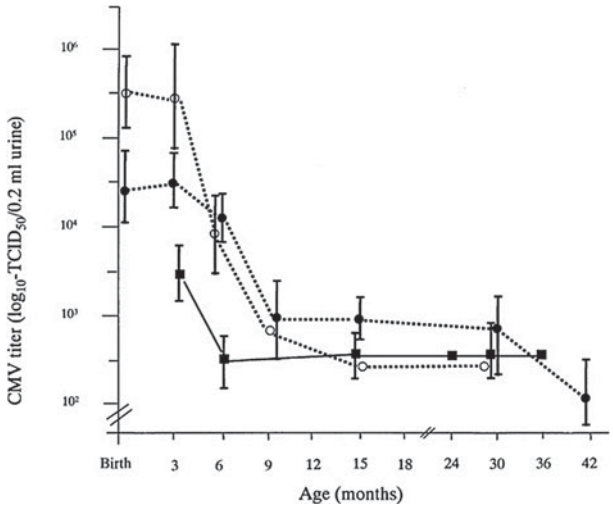

FIGURE 7 CMV load in the urine of neonates. $TCID_{50}$, 50% tissue culture infective dose. Symbols: ○, symptomatic congenital infection; ●, asymptomatic; ■, natal infection. Error bars indicate standard errors of the means.

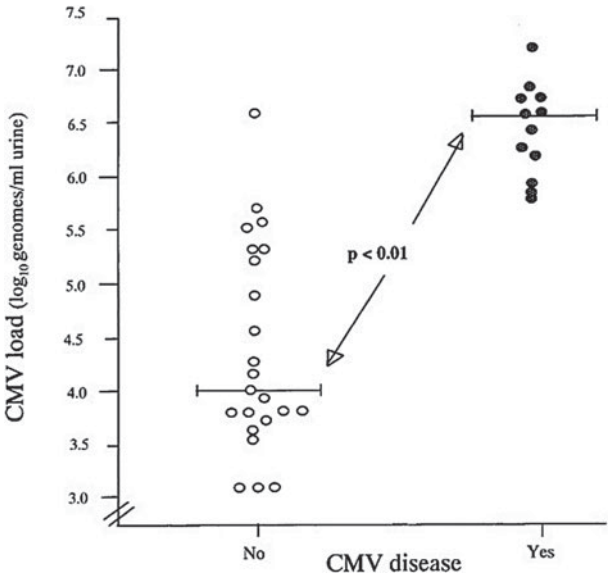

FIGURE 8 CMV load in the urine of renal transplant recipients.

patients (107). For example, in renal transplant patients, the peak level of viruria and risk of HCMV disease are clearly related ($P < 0.01$) (Fig. 8). The difference between the median values in those with and without disease is approximately 2 logs, very similar to the difference found in the neonates using cell culture methods (90). Among three factors associated with HCMV disease (i.e., high viral load, viremia, and recipient serostatus), only viral load remains a significant independent risk factor in a multivariate model (Table 6). Furthermore, a plot of peak viral load against the probability of HCMV disease has a sigmoidal shape (Fig. 9), showing that HCMV infection is well tolerated clinically until very high levels are reached. Viral load measurements in stored neonatal dried blood spots from children who subsequently developed hearing loss have also confirmed the threshold relationship (108). This sigmoidal relationship suggests that antiviral interventions and vaccination strategies should be designed to prevent viral loads rising into the critical high range of $>10^3$ to 10^4 genomes/ml of whole blood (or 10^2 genomes/semicircle of dried blood spot). Furthermore, the shape of the curve implies that a marked effect on HCMV disease could be achieved with a modest reduction in peak viral load. In AIDS Clinical Trials Group protocol 204, in which 1,227 AIDS patients were randomized to receive valacyclovir at 2 g daily or one of two doses of acyclovir, the higher of which had been shown to be ineffective in controlling HCMV disease, it was found that valacyclovir significantly reduced HCMV disease despite a relatively modest reduction in HCMV load from baseline (median, 1.3 logs) (109). The serial measures of virus load in blood have also revealed the dynamic process of HCMV replication in its host, with an average doubling time (viral load increasing) or half-life (viral load decreasing) of approximately 1 day (110). Furthermore, serial measures of viral load can provide estimates of the efficacy of antiviral drugs, such as ganciclovir, required to bring HCMV replication under control (111).

TABLE 6 Univariate and multivariate assessment of prognostic factors for HCMV disease in renal allograft recipients[a]

	Univariate[a] factor			Multivariate[a] factor		
Parameter	OR	95% CI	*P*	OR	95% CI	*P*
Viral load (per 0.25 log)	2.79	1.22–6.39	0.02	2.77	1.07–7.18	0.04
Viremia	23.75	3.69–153	0.0009	34.54	0.75–1599	0.07
R^+ serostatus	0.22	0.05–0.95	0.05	0.92	0.002–446	0.98

The results demonstrate that virus load is a major determinant of disease and that viremia and recipient serostatus are markers of disease because of their statistical association with high viral load.

[a]OR, odds ratio; CI, confidence interval.

Differences Among Groups of Immunocompromised Patients

Table 7 provides an overview of the relative importance of HCMV disease in different patient groups. The phrase "HCMV infection in the immunocompromised host" obscures the great diversity among the various patient groups. The pathogenesis of bone marrow failure after bone marrow transplantation is thought to follow HCMV infection of stromal cells releasing cytokines and so producing a milieu unfavorable for hematopoiesis (112).

CMV Pneumonitis

HCMV pneumonitis after bone marrow transplantation may be immunopathologically mediated (102). This hypothesis explains the timing of pneumonitis, because it occurs only after patients have engrafted their bone marrow, arguing that a host immune response is required for disease. It also explains why AIDS patients do not appear to suffer from HCMV pneumonitis, although HCMV is frequently found in their lungs. Presumably they cannot mount the immunopathological response required to cause disease. Patients with HIV seroconversion illness may, however, get HCMV pneumonitis, presumably because their cell-mediated immune response has not been sufficiently abrogated at this stage (113). The precise nature of the immunopathological response has not been identified, although the histologic appearance (Fig. 10) suggests that it is probably mediated by cytokines attracting leukocytes to the lung interstitium and so increasing the distance required for gas diffusion from alveoli to blood. If so, it is perhaps surprising that cases of HCMV pneumonitis have not been seen in patients given highly active antiretroviral therapy (HAART), in whom the regenerating immune system could recognize HCMV-infected target cells and trigger immunopathology. Perhaps HAART provides protective cell-mediated immune responses able to control HCMV replication in the lungs.

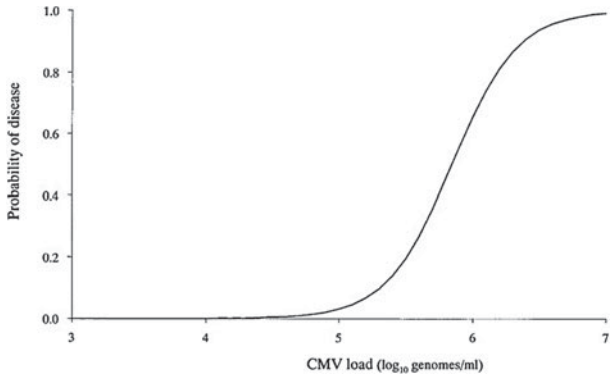

FIGURE 9 Threshold concept of CMV pathogenesis.

TABLE 7 HCMV diseases in immunocompromised persons

Symptom	Symptom in patients with[a]: Solid-organ transplant	Bone marrow transplant	AIDS
Direct effects			
Fever/hepatitis	++	+	+
Gastrointestinal	+	+	+
Retinitis	+	+	++
Pneumonitis	+	++	
Myelosuppression		+	
Encephalopathy			+
Polyradiculopathy			+
Addisonian			+
Indirect effects			
Immunosuppression	+		
Rejection/GVHD	+	?	
Atherosclerosis	+		
Death		+	+

[a]+, symptom occurs; ++, symptom most common.

CMV Retinitis

The dramatically increased proportion of HCMV retinitis found in AIDS patients compared to transplant patients remains unexplained but may result from prolonged exposure to high levels of viremia. Another new disease has occurred in AIDS patients given HAART that has all the hallmarks of immunopathology. Patients with a history of HCMV retinitis, which is typically now clinically silent, can nevertheless develop an inflammatory vitritis due to the infiltration of $CD8^+$ T cells specific for HCMV.

CMV Encephalitis

In AIDS patients, viremia can disseminate HCMV to the brain in two distinct ways. The first seeds virus to the brain endothelial cells, allowing HCMV to spread to contiguous astrocytes and then to neurons. The histopathologic correlates of this route of infection include endothelial cell infection/inflammation, with or without thrombosis, and multiple areas of glial nodular encephalitis (114). The second seeds virus to the choroid plexus epithelial cells and thence to the cerebrospinal fluid (CSF). Transmission to ependymal surfaces produces necrotizing ventricular encephalitis.

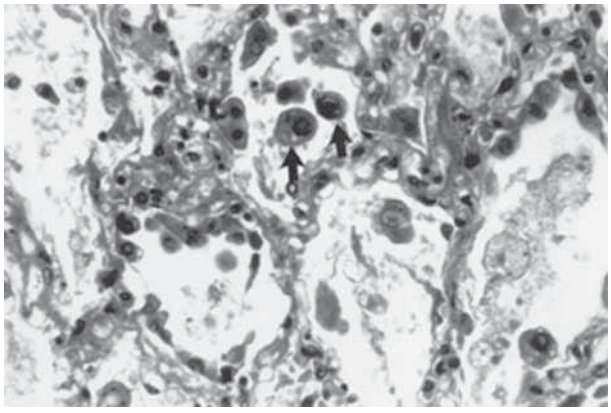

FIGURE 10 Histologic section of a lung sample from a patient with HCMV pneumonitis following bone marrow transplantation. Arrows show alveolar macrophages bearing the typical intranuclear inclusions of HCMV. An interstitial mononuclear cell infiltrate is seen.

CMV Polyradiculopathy

Viremic dissemination seeds CMV to peripheral nerves and the cauda equina.

Gastrointestinal Involvement

Viremic dissemination leads to focal ulceration anywhere in the gastrointestinal tract but often in the esophagus or colon. In patients with inflammatory bowel disease, extensive ulceration may mimic relapse of the underlying disease and be exacerbated by steroid administration. This is consistent with inflammation providing an environment that supports CMV replication.

Other Syndromes

In addition to these end-organ diseases (EODs), HCMV is associated with a variety of conditions collectively called indirect effects (115). Although HCMV has been shown for many years to be associated with these conditions, it is only recently that the results of controlled clinical trials have demonstrated that the associations are causal. The precise mechanisms whereby HCMV causes these effects have not been demonstrated, although there are several candidates, not least of which is the possibility that HCMV perturbs normal immune functions. In addition, in the case of graft rejection, HCMV may act like a minor transplantation antigen, may up-regulate cellular class I molecules bearing donor epitopes in adjacent non-infected cells, or may express the UL18 class I homolog. Fungal superinfection may reflect HCMV-induced immunosuppression, although there is no immunologic marker of the same. Finally, HCMV may trigger post-cardiac allograft coronary atherosclerosis because (i) pUS28 confers chemotactic mobility on smooth muscle cells; (ii) IE72 binds p53 in the same cells, preventing their apoptosis; or (iii) HCMV activates cyclooxygenase, so producing reactive lipoprotein peroxides.

In AIDS patients, clinically silent HCMV infection may similarly interact in multiple ways with HIV to increase its pathogenicity (i.e., act as a cofactor for progression of HIV disease) (116). This subject is controversial but is supported by data showing that HCMV can transactivate the HIV long terminal repeat *in vitro* and can provide an alternative receptor for HIV by inducing an Fc receptor (117). HCMV is widely disseminated at autopsy and is found in most tissues (Table 4). HCMV-HIV coinfection of individual cells or organs has also been described. The risk of AIDS is increased among male homosexuals with persistent HCMV excretion in semen, even after allowing for differences in baseline CD4 counts (118). Cohorts of patients develop AIDS more rapidly if they are coinfected with HCMV. Furthermore, high levels of HCMV viremia are significantly associated with the death of AIDS patients (119). This effect is stronger than the association of HIV viral load with death (120) and persists in the era of HAART (121). Patients given intraocular treatment for HCMV retinitis had a survival benefit if they were also given systemic treatment (122). Overall, it is difficult to avoid the conclusion that HCMV adversely affects the outcome in AIDS patients but that this effect is clinically inapparent and can only be documented by appropriate laboratory studies. Overall, these results are consistent with the possibility that HCMV may play a cofactor

role, but extensive controlled investigations would be required to establish this conclusively.

Immune Responses

Herpesviruses persist by hiding from immune responses rather than by blocking their induction. Thus, all of the proteins encoded by HCMV are recognized by the host, so the human immune system commits more resources to controlling HCMV than to controlling any other virus (9). The major targets for CD8 T cells against HCMV are ppUL83 (pp65) and the IE2 protein, and the frequency of responding CD8 T cells against HCMV is very high. For example, using class I HLA tetramers in immunocompetent and immunocompromised patients with or without active replication, frequencies of CD8 T cells against epitopes within pp65 are approximately 1% in healthy seropositive individuals and up to 10% in patients experiencing active HCMV replication (123).

Prior exposure to HCMV can ameliorate the pathological potential of HCMV in some, but not all, patients. In pregnant women, the risk of damage to the fetus and the severity of that damage are clearly decreased in women who were "immune" before they became pregnant (124), suggesting that prior immunity in the mother can reduce the extent of HCMV exposure to the fetus. In women experiencing primary infection during pregnancy, an increased titer of neutralizing antibody and its avidity correlate with reduced risk of disease in the neonate (87).

The results for bone marrow transplant patients are distinct because reactivation of the recipient virus represents the source of HCMV-causing disease. Nevertheless, among seropositive recipients, those receiving marrow from immune donors have a reduced risk of disease (95). Direct evidence for adoptive transfer of immunity from the donor into the recipient was found by immunizing donors or recipients (or both, or neither) with tetanus toxoid (125). Although cell-mediated immunity is clearly the major response capable of containing HCMV infection, the possibility that HCMV disease may be controlled by other responses such as humoral immunity has been incompletely investigated. Humoral immunity could reduce the level of HCMV replication and reduce disease without being able to eliminate infection entirely. Humoral immune responses are directed against multiple CMV proteins, including surface glycoproteins, phosphoproteins of the tegument, and structural proteins of the capsid. Much of the antibody that neutralizes infectivity *in vitro* can be absorbed from sera with recombinant soluble gB, showing that this is the major target of the neutralizing response, but additional neutralizing epitopes are also found on gH, gL, and gN.

CLINICAL MANIFESTATIONS

Congenital Infection

Based on a systematic review of published prospective case series (85) 12.7% of congenitally infected babies are born with "cytomegalic inclusion disease" (Table 8). The remaining 87.3% appear to be normal at birth, but a proportion develop sequelae on follow-up, as described below.

In those symptomatic at birth, most of the noncentral nervous system (non-CNS) disease is self-limiting, although severe thrombocytopenic purpura, hepatitis, pneumonitis, and myocarditis may occasionally be life threatening (Table 8). A total of 0.5% of these patients die during infancy, and 50% of the survivors have permanent serious abnormalities. Brain damage may present as microcephaly, mental retardation, spastic diplegia or seizures, and perceptual organ damage such as optic atrophy, blindness, or deafness. These abnormalities may occur alone or in combination.

TABLE 8 Clinical features of cytomegalic inclusion disease

Intrauterine growth retardation
Jaundice
Hepatosplenomegaly
Thrombocytopenia
Microcephaly
Intracranial calcifications
Retinitis

Among those asymptomatic at birth, approximately 13.5% will develop hearing defects or impaired intellectual performance (Table 9). Twice as many children who come from the group who appear to be normal at birth are ultimately damaged by congenital HCMV, compared to those born with classical symptoms. This helps to explain why the burden of disease is underestimated for this infection.

A plausible pathogenesis for progressive hearing loss is apparent from the histologic examination of inner ear structures from a patient with a fatal case of congenital HCMV infection (Fig. 11). The virus spreads by the cell-to-cell route to produce a focus of infection surrounded by inflammation. This may represent stages of the infectious process in the inner ear, explaining how progressive damage to the organ of Corti could occur.

Perinatal Infection

Neonates acquiring perinatal infection can start to excrete CMV in the urine from 3 weeks of age onwards. Most perinatally infected infants do not develop acute symptoms, although occasional cases of infantile pneumonitis develop, making HCMV a frequent pathogen in those few children presenting with an appearance of sepsis in the first 3 months of life (126). Perinatal infection may be severe in premature neonates because they do not benefit from transplacental passage of maternal immunoglobulin G (IgG) antibodies.

Postnatal Infection

Primary infection in the immunocompetent child or adult is almost always asymptomatic, except for occasional cases of infectious mononucleosis. The patient presents with a fever with few localizing symptoms; pharyngitis, lymphadenopathy, and splenomegaly are less common than in EBV

TABLE 9 Disease outcome in 1,000 infants born with congenital HCMV infection[a]

Outcome	No. (%)
Born with symptoms of CID[b]	127 (12.7%)
Die during infancy	5 (0.5%)
	122
50% develop long-term handicap	61
Born without symptoms of CID	873
Develop handicap	118 (13.5%)
Total damaged	179 (18%)

[a]Adapted from reference 87.
[b]CID, cytomegalic inclusion disease.

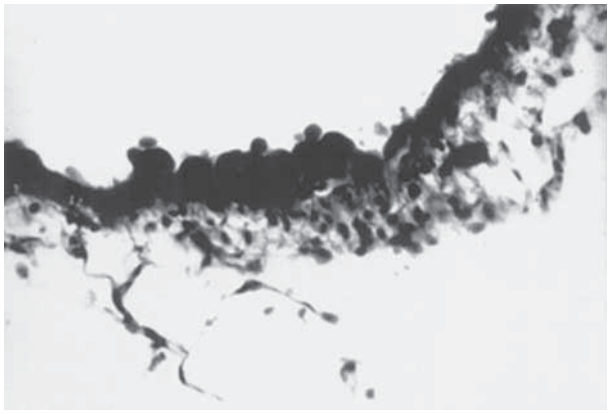

FIGURE 11 Histologic appearance of the inner ear in a patient with a fatal case of cytomegalic inclusion disease. Note the focus of large inclusion-bearing cells and accompanying inflammation. (Courtesy of S. Stagno. Reprinted from reference 188 with permission.)

mononucleosis. Laboratory tests reveal biochemical hepatitis, with moderately raised transaminases, lymphocytosis with atypical mononuclear cells, and a negative heterophile agglutinin test. The condition resolves spontaneously, with a mean of 19 days' fever in one large study (127). Guillain-Barré syndrome has been described as a complication of postnatal primary HCMV infection. Rare cases of CMV EOD have been described among patients who are immunocompetent, including hepatitis, gastrointestinal ulceration with or without hemorrhage, and pneumonitis. Presumably these individuals have received a larger-than-average inoculum of CMV to explain their severe outcome.

Immunocompromised Patients

Active HCMV infection causes a wide spectrum of disease, ranging from life threatening to asymptomatic and involving specific organs or causing constitutional disturbances.

Fever is a common component of all HCMV diseases in all immunocompromised hosts. Typically, this follows a spiking pattern, with temperatures in the range of 38 to 40°C, followed by precipitous declines below 37°C. During the fever, the patient complains of malaise and lethargy and may develop myalgia or arthralgia. This systemic phase of HCMV with leukopenia and elevated liver transaminases may resolve spontaneously or may herald particular clinical syndromes (EOD due to viremic dissemination of virus) that vary in incidence according to the underlying cause of the immunocompromised state (Table 7). The most common clinical presentations for each patient group are illustrated in Table 7, but any can occur in any patient group. HCMV disease typically manifests when the patient is most profoundly immunocompromised, that is, in the first and second months post-transplantation or, in AIDS patients, once the CD4 count has declined below 50 cells/μl. Indeed, successful institution of HAART reverses these changes and leads to immune-mediated control of HCMV viremia and prevention of EOD.

Recipients of Solid-Organ Allografts

Leukopenia is common and may be profound. If leukopenia persists, it is associated with the development of secondary fungal and bacterial infections (128). Biochemical evidence of hepatitis is often found, with transaminase levels raised two to three times the upper limit of normal. Thrombocytopenia may occur, with serial daily platelet counts below 100,000. All of these features usually respond to antiviral therapy.

Pneumonitis with interstitial infiltrates may occur, especially in recipients of lung (or heart-lung) transplants. Despite prolonged treatment, obliterative bronchiolitis may supervene (129), showing that HCMV plays an important role in the etiology of this chronic rejection process. Likewise, HCMV has been implicated in the development of accelerated atherosclerosis after cardiac transplantation (130) or graft rejection and graft atherosclerosis (131, 132). These conditions present as dysfunction of the transplanted organ with no clinical symptoms or signs to reveal the underlying contribution from HCMV. Yet, approximately 50% of acute biopsy-proven renal allograft rejections can be prevented by anti-HCMV prophylaxis with valacyclovir (133). Thus, the conventional diagnostic conundrum in the febrile allograft recipient of "rejection or HCMV" should be replaced with "rejection and HCMV."

Recipients of Bone Marrow Grafts

Pneumonitis is the major life-threatening presentation of HCMV, occurring in 10% to 20% of bone marrow allograft patients before the availability of antiviral drugs. The patient presents with fever and hypoxia associated with pulmonary interstitial infiltrates. This complication is much less common (less than 5%) after an autograft, consistent with a postulated immunopathological disease process. HCMV infection may delay marrow engraftment (112) by replicating in bone marrow stromal supporting cells. An important clinical feature is that HCMV disease, especially pneumonitis, is statistically associated with GVHD (134). It is not clear whether HCMV infection can precipitate GVHD or whether the immunosuppressive nature of GVHD, or the treatment required for its suppression, facilitates HCMV reactivation.

Patients with AIDS

In the pre-HAART era, at least 25% of AIDS patients developed disease attributable to HCMV. The vast majority (85%) of cases had retinitis, a clinical manifestation that is rare in transplant patients, followed by gastrointestinal involvement, encephalitis, and polyradiculopathy.

A patient with CMV retinitis may complain of "floaters" or loss of visual acuity. Alternatively, a typical focus of retinitis may be recognized at routine follow-up visits. Early lesions may be white due to edema, necrosis, or both (see figures in Chapter 10). Without treatment, the focus of infection spreads to neighboring cells (135), leaving white necrosis at the advancing border. There is hemorrhage, often flame shaped, surrounding blood vessels, with or without perivascular sheathing. It may be accompanied by anterior uveitis, retinal edema, or retinal detachment (136).

HCMV may also involve the gastrointestinal tract by causing ulcers deep in the submucosal layers. Clinical features vary with the anatomic site involved. Odynophagia is a common presentation of HCMV esophagitis, whereas abdominal pain and hematochezia frequently occur with HCMV colitis. Ulceration at these sites may cause perforation or hemorrhage.

HCMV causes encephalitis of two types in AIDS patients that mirror the pathology described above. The first is difficult to differentiate clinically from HIV dementia and manifests as subacute or chronic symptoms of confusion and disorientation attributable to cortical involvement. Focal

signs can be attributed to lesions in the brain stem. The second manifests as defects in cranial nerves, nystagmus, and increasing ventricular size (137), which progress rapidly to a fatal outcome.

HCMV also causes polyradiculopathy. Patients subacutely present with weakness of legs and numbness, progressing to flacid paraparesis, often with pain in the legs and perineum, bladder dysfunction, or both. The CSF shows a remarkable preponderance of polymorphonuclear leukocytes.

In the HAART era, most of these HCMV diseases have become uncommon (136). Nevertheless, HAART- induced immune recovery can produce vitritis and cystoid macular edema. These both profoundly affect vision, as does retinal detachment, which occurs when an HCMV-infected retina loses its substratum attachment to the underlying choroid layer (136).

The Apparently Healthy Elderly

Immunosenescence, defined as a reduced T-cell CD4/CD8 ratio, is a major underlying contributor to morbidity and mortality (reviewed in reference 138). Immunosenescence is significantly increased in HCMV seropositive elderly persons compared to seronegative controls, although there are no differentiating clinical features between the two groups. However, HCMV seropositivity represents a significant risk factor for imminent death in the elderly (138). Immunosenescence is characterized by an excess of clones of differentiated T cells, detected by their restricted Vβ repertoire, which have a reduced ability to respond to new antigens. (139, 140). Many clones of differentiated cells are HCMV specific, have an activated phenotype, and so may contribute to chronic inflammatory conditions, including atherosclerosis. Direct evidence for the adverse functional consequences of HCMV-associated immunosenescence include 2 possibilities; a decrease in naive T cells required to respond to new infections and vaccines; an increase in activated T cells that could contribute to chronic inflammation (140). T cells with the phenotypic markers of immunosenescence are found also in patients with chronic HIV infection where a randomized controlled trial showed that valganciclovir significantly reduced their abundance (141). To date, there are no randomized controlled trials of antiviral drugs or of immunotherapeutic vaccines in the elderly.

LABORATORY DIAGNOSIS

Detection of Virus

The characteristics of the assays used frequently in the identification of active HCMV infection vary widely among laboratories (Table 10). While the precise methodological details vary, all diagnostic virology laboratories should offer a service for rapid HCMV detection. The clinician should inquire about the positive and negative predictive values of particular tests, understand their interpretation, and review whether day-to-day results have been audited to ensure that appropriate targets are achieved.

Specimen Selection

Urine or saliva samples are usually collected to diagnose congenital or perinatal infection. If lumbar puncture or liver biopsy is indicated clinically, then samples can be processed, but the detection of HCMV at these sites does not have prognostic value for these children.

For adults with mononucleosis or hepatitis, urine and blood are the best samples. HCMV viruria might be coincidental, but the detection of viremia strongly supports the diagnosis. Pregnant women with symptoms should be investigated as for other adults. The screening of asymptomatic women has not been shown to identify those who will have newborns with congenital infection and so is not recommended.

Transplant patients should have surveillance samples of blood taken at least weekly from the time of transplantation. In the past, urine and saliva samples were also collected, but this has been superseded by assays using blood. Whole blood is the most sensitive for PCR (142). HCMV excretion from urine and saliva is very common but only doubles the relative risk for future disease. In contrast, viremia is detected less frequently in allograft patients but increases the relative risk to about 6 (Table 5). These different risks are used to define distinct treatment strategies discussed below.

The basic principles of HCMV natural history in patients who are HIV positive are the same as in the pre-HAART era, and these principles may become relevant again as individuals exhaust the salvage antiretroviral options in the post-HAART era. Individual AIDS patients with suspected CMV infection can be investigated for CMV viremia and by examining CSF by PCR when encephalitis or polyradiculopathy is suspected.

Laboratory Assays

Assays to Detect HCMV in the Blood

Real-time PCR has become the new "gold standard" for detection of HCMV, in part because cell culture results can be misleading (Table 11). Because HCMV persists for the lifetime of infected individuals, a very sensitive technique such as nested PCR could potentially detect latent HCMV or virus that was replicating at such a low level that it had no clinical consequences. Four approaches have been taken to avoid this disadvantage.

Minimization of Latent Viral DNA Detection

A non-nested PCR method with high sensitivity but using only a small quantity of sample nucleic acid for analysis (i.e., 5 µl of urine or 30 ng of DNA from peripheral whole

TABLE 10 HCMV detection in body fluids

Method	Sensitivity	Specificity	Reliability	Rapidity	Proven prognostic value
Conventional cell culture	++	+++	++		+
Detection of early-antigen fluorescent foci (shell vial)	+	+++	++	+	+
Antigenemia	++	+++	++	++	++
PCR	+++	+++	++	+	+++

TABLE 11 Misleading concepts about HCMV derived from propagation of the virus in fibroblast cultures

Concept	Fact	Reference(s)
In vitro strains are genetically representative of those found *in vivo*	Strain Ad169 has 22 missing ORFs; Towne has 19 missing ORFs	31
A live attenuated vaccine can be prepared in fibroblasts	No protection against HCMV infection from Towne strain, although severity of disease is reduced	154
In vitro assays correctly identify the susceptibility of HCMV to antivirals	Failed to detect clinically important susceptibility to acyclovir	133
HCMV is a slowly replicating virus	HCMV replicates rapidly	110
Strains of HCMV resistant to ganciclovir occur infrequently in immunocompromised patients	Resistance is more common; cell cultures select against detection of resistant strains	111

blood) has correlated well with conventional cell culture and provided useful prognostic information (Table 5) (143).

Plasma PCR

Because the latent DNA of HCMV persists in the cellular fraction of blood, the detection of HCMV DNA in plasma should reflect active infection, although in practice some viral DNA is released from leukocytes prior to separation of the serum (99). An FDA-approved commercial assay is available (Roche Amplicor).

RT-PCR and NASBA

Reverse transcription-PCR (RT-PCR) should detect only cells in which HCMV is replicating, since the target is mRNA. A nucleic acid sequence-based amplification (NASBA) assay for the detection of a late gene transcript (pp67) appears to be sensitive and specific and is available commercially (Biomerieux Nuclisens). However, there is no evidence that this assay offers any advantages over standard PCR.

Real-Time PCR

Quantitative assessments of HCMV load are possible in real time. While such assays usually do not incorporate quantitative-competitive formats to identify possible inhibitors of PCR in the clinical sample, the results represent an important contribution toward controlling HCMV disease. The viral load found in the first available sample correlates with the peak viral load (144). The rate of increase in viral load can be estimated by back-projecting from the initial load to the last available PCR-negative sample from that patient. In multivariate models, the parameters of initial viral load and rate of increase are independent of each other, so both parameters can be used in combination to estimate an individual's risk of future HCMV disease (144). Serial results from real-time PCR are frequently used to decide when to initiate preemptive therapy and to monitor the response to this treatment. In addition to testing blood, PCR has become invaluable for the investigation of CNS involvement in AIDS patients.

Antigenemia

Monoclonal antibodies can be used to detect HCMV antigens directly in leukocytes from the blood of immunocompromised patients. The monoclonal antibodies react with pp65, the product of UL83. These monoclonal antibodies stain polymorphonuclear cells, monocytes, and endothelial cells from the peripheral circulation. The advantages of this technique are that it takes only a few hours and does not require facilities for cell culture or for PCR. The disadvantages are that it is subjective and samples can deteriorate rapidly. The antigenemia assay is therefore used by laboratories situated close to clinical facilities. It is possible to count the stained cells to provide a semiquantitative assessment of viral load. In general, antigenemia has been replaced by real-time PCR in most laboratories.

Histopathology

Biopsies are often taken to confirm organ involvement. HCMV is recognized by its characteristic intranuclear "owl's eye" inclusions, which have a surrounding halo and marginated chromatin. They occur in kidney tubules, bile ducts, lung and liver parenchyma, and the gastrointestinal tract, inner ear, and salivary gland but are less prominent in brain tissue (Figs. 10 and 11). Cytoplasmic inclusions can also be seen in infected cells, particularly when stained with CMV-specific monoclonal antibodies. Histopathology provides a specific diagnosis that is insensitive (Fig. 12) compared to viral load measurements made by PCR in the same organs (145).

Virus Isolation

Human fibroblasts (typically from foreskins or embryo lungs) are used to support HCMV replication *in vitro*. For the detection of viremia, buffy coat or unseparated heparinized blood can be inoculated into cell cultures. If toxicity is observed, denuded areas of the monolayer can be repaired by the addition of fresh fibroblasts.

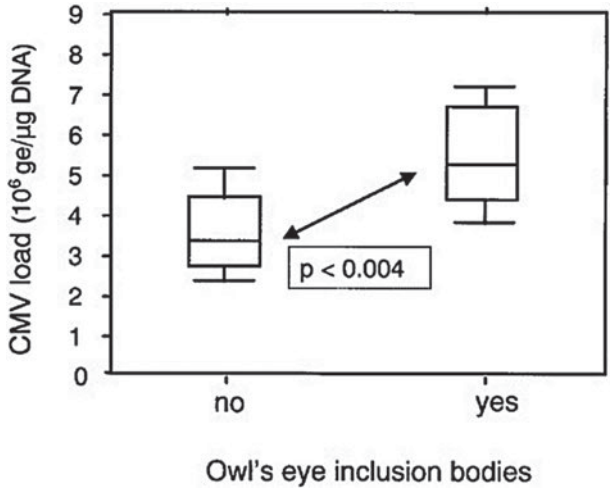

FIGURE 12 Correlation between high HCMV load and detection of intranuclear inclusions. ge, genomes.

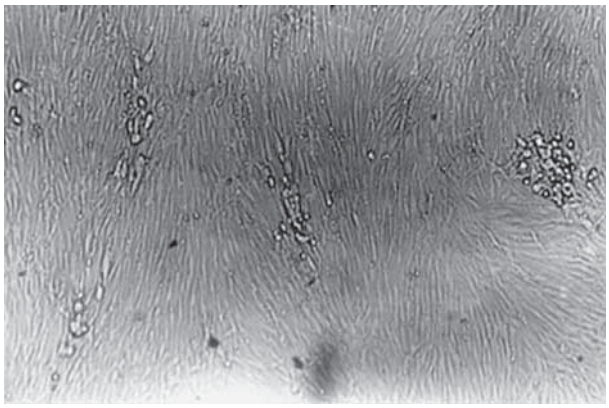

FIGURE 13 Photograph of human embryonic lung fibroblasts showing the focal CPE of HCMV. (Figure prepared by J. A. Bishop.)

All cultures should be observed at least twice weekly for the typical focal CPE of HCMV (Fig. 13). Occasionally, urine samples from patients with congenital infection produce widespread CPE within 24 to 48 hours that resembles that of HSV. More usually, the CPE evolves only slowly, typically becoming apparent at 14 to 16 days, so the cultures must be maintained for a minimum of 21 days before being reported as negative.

Monoclonal antibodies against the major IE and other E proteins of HCMV provide reagents useful in detecting the virus in cell cultures within 48 h before CPE had become apparent. Two techniques, (146) the shell vial assay (after the name of the container), and the (147) DEAFF test (detection of early antigen fluorescent foci) were a great improvement in their day due to the rapid availability of results. They have now been replaced by real-time PCR (see above) for testing surveillance samples but are still used to diagnose congenital infection (148, 149).

Serologic Assays

IgG Antibody

Many assays can detect HCMV IgG antibodies. The presence of IgG antibodies against HCMV is indicative of infection sometime in the past. Because the seropositive individual is liable to experience reactivations of his or her latent infection, the presence of IgG antibodies against HCMV is a marker of potential infectivity and immunologic responses do not imply complete protection from endogenous or exogenous infection.

IgG antibody detection can also be used as a marker of recent infection in populations, such as pregnant women, in whom the availability of avidity assays offers a practical way of detecting asymptomatic primary HCMV infections. Typically, it takes 16 to 20 weeks for IgG avidity to mature to high levels, and the reproducibility and specificity of the commercial assays appear to be superior to those of IgM assays. For pregnant women found on routine screening to be IgM positive, avidity assays can be used to triage them into groups with primary infection or recurrent infection and so reduce the number of terminations of pregnancy that might be undertaken based on the results of IgM testing alone (150). These tests are best performed in specialist laboratories with access to a range of commercial and in-house assays (150).

To detect serologic responses due to native virus infection after immunization with candidate vaccines, sera can be preabsorbed with the immunogen to ensure that responses to this component of the vaccine are not mistaken for seroconversion (151).

Immunocompromised patients most at risk of HCMV disease may be those least able to mount prompt immune responses. In addition, false-positive *interpretations* are frequently seen when patients receive blood or blood products. Note that these are not false-positive *reactions*, as the patients do truly have HCMV IgG antibodies in their blood but these have come from an exogenous source. Thus, serial serologic testing is not recommended for monitoring individual immunocompromised patients.

IgM Antibody

HCMV-specific IgM antibodies can also be used to detect current infection in immunocompetent patients such as those with HCMV mononucleosis. However, the specificity is poor for screening pregnant women for primary infection. The serologic approach is not recommended for the immunocompromised individual, in whom rapid detection of CMV is preferred.

Detection of Antiviral Resistance

The protein kinase enzyme encoded by UL97 phosphorylates ganciclovir to its monophosphate. Cellular enzymes then convert this to the active triphosphate which is an inhibitor of the virus- encoded DNA polymerase UL54. HCMV can become resistant to ganciclovir through mutations in UL97 or, more rarely, UL54. Polymorphisms also occur in both genes, so a genetic change from that expected cannot, on its own, be taken as evidence of resistance. Instead, mutations should be transferred to a laboratory strain and shown to confer resistance *in vitro*.

Genetic changes in UL97 proven by site-directed mutagenesis to confer resistance when introduced back into a laboratory-adapted strain are shown in Fig. 16. These changes are detected by PCR amplification from clinical samples followed by sequencing. Many changes are clustered within specific regions of the gene so that small sections can be amplified by PCR to provide results rapidly. Changes in UL97 do not confer cross-resistance to any currently available anti-CMV drug. Unlike the situation with UL97, a number of mutations have been detected throughout the UL54 ORF following *in vitro* passage in the presence of antiviral drugs and in strains derived from patients undergoing drug therapy. Sequence analysis of the entire ORF is thus required to allow the detection of drug resistance at this locus in a clinical setting. Some changes in UL54 selected through use of ganciclovir confer cross-resistance to cidofovir (and brincidofovir) while rare changes are cross-resistant to foscarnet. None of these changes confers cross-resistance to maribavir or letermovir.

PREVENTION

Because of the risk of fetal damage in pregnant women, especially when seronegative, changes in child-rearing practices could affect the incidence of disease attributable to congenital HCMV. Typically, middle-class seronegative women are at potential risk when they send their children to child care centers with young children excreting HCMV (90). Considerable anxiety may be induced in both mothers and day care workers because HCMV infection is usually asymptomatic and excretion is prolonged. Because professionally trained nursing staff are not at increased risk of acquiring HCMV infection from patients (91), routine

precautions such as hand washing must be sufficient to protect against transmission. Consequently, day care centers should be advised to review and improve their general hygiene standards. The same advice should be given to female staff, irrespective of their serologic status; humoral immunity in these women cannot guarantee that the fetus will be protected from infection, although it should reduce the chance of disease.

HCMV infection can be prevented in some patients by screening of blood products. Blood from donors who are seronegative should ideally be used for intrauterine transfusion, for pregnant women, and for immunocompromised patients, irrespective of whether the recipients are CMV seronegative or seropositive. Because such blood is in short supply, an alternative is to pass the blood through in-line filters capable of retaining leukocytes (152).

In the solid-organ transplantation setting, HCMV disease could be potentially reduced by matching donors and recipients, so that organs from seropositive donors are not given to seronegative recipients. However, in practice, this would reduce the likelihood of achieving the best available HLA match and would delay transplants for many recipients whose medical condition may deteriorate while they wait for a seronegative donor. Furthermore, because donor organs are in such short supply, there is pressure not to reject donations unless absolutely necessary. As a result, donor-recipient matching is generally required only by some centers where the risk for HCMV disease is considered especially high, such as for seropositive donors of lungs destined for seronegative recipients. In the remaining patients, various antiviral strategies can be employed to reduce HCMV disease.

Active Immunization

No approved HCMV vaccine is currently available. A summary of HCMV vaccine candidates is shown in Table 12, and a list of possible volunteers for immunogenicity and phase III protection studies is shown in Table 13.

Pioneering work with live-attenuated vaccine strains by Plotkin and colleagues showed in volunteers that the Towne strain was truly attenuated compared to the virulent Toledo strain (153). A controlled trial in dialysis patients showed no reduction in the proportion of patients who became infected or ill due to CMV after subsequent renal transplantation, but the trial demonstrated a significant reduction in the severity of disease (154). The Towne vaccine did not provide protection against primary infection in parents of children attending day care centers. Overall, the effects seen were not sufficiently encouraging to allow further development of this vaccine but provided a useful framework for the evaluation of future preparations.

TABLE 12 Vaccine candidates studied clinically

Preparation	Type	Status
Towne	Live attenuated	Phase II
gB	Soluble recombinant	Phase II; 3 trials
Towne/Toledo recombinant	Live attenuated	Phase I
Canarypox virus pp65	Live, single cycle, heterologous	Phase I
pp65, gB	DNA plasmids	Phase III
Alphavax	Live, single cycle, heterologous	Phase I
City of Hope	Peptides	Phase I
Whole virus	Replication-defective	Phase I

Phase I studies are under way to administer Towne vaccine together with IL-12 as a way of improving immunogenicity. The Towne strain of HCMV lacks 19 genes found in the Toledo strain (31), and it is possible that some of these genes could provide protection against wild-type HCMV if they were incorporated into a vaccine. Accordingly, recombinants between Towne and Toledo have been prepared that include all 19 genes incorporated into the Towne attenuated background. Vaccine candidates containing all 19 genes have shown safety and immunogenicity in a phase I study.

Recombinant vaccines based upon gB have been developed, because gB can adsorb most of the neutralizing antibody from sera and also contains T-helper epitopes. The gB also possesses T-helper epitopes, although humans with particular HLA types have been shown to be low responders. Cells infected with a vaccinia virus gB recombinant adsorb between 40% and 88% of the total serum neutralizing activity in individuals who are either naturally immune or vaccinated with the Towne vaccine strain of HCMV. Purified and recombinant preparations of gB induce humoral immunity in experimental animals and can reduce the fetal loss and congenital infection that result from inoculation of guinea pig HCMV.

A canarypox virus vector that incorporates the ppUL83 (pp65) gene, which is the major target for cell-mediated T-cytotoxic responses, was immunogenic in phase I studies, although no humoral immunity was induced. Phase I trials of a DNA vaccine for HCMV in humans provide evidence of priming of the immune system. An alphavirus recombinant expressing gB has been shown to be well tolerated and immunogenic in a phase I study (Table 12).

Most clinical experience to date has been with a truncated form of gB expressed in mammalian cells and used to immunize volunteers in phase I and phase II studies. This prototype vaccine was immunogenic in seronegative healthy volunteers and induced neutralizing antibody titers greater than those found in seropositive persons (155). The novel adjuvant MF59 gave antibody titers superior to those obtained with the conventional adjuvant, alum, and the optimum immunization schedule was to give vaccine at 0, 1, and 6 months (155). Although antibody levels declined with time after this primary course of three vaccine doses, a prompt anamnestic response to a booster dose given at 12 months was seen (155). This vaccine produced very high neutralizing titers when given to seronegative toddlers and also boosted the titer of neutralizing antibodies when given to seropositive individuals. This gB/MF59 vaccine underwent 3 phase II studies. A controlled study in postpartum seronegative women determined that the rate of primary maternal infection was decreased by approximately 50% (156). Seronegative adolescents and seronegative or seropositive recipients of a kidney or a liver transplant also had evidence of reduced CMV infection when exposed to virus (157).

A summary of HCMV vaccine candidates is shown in Table 12, and a list of possible volunteers for immunogenicity and phase III protection studies is shown in Table 13. One theoretical concern that has been raised about widespread introduction of an HCMV vaccine is that alterations in herd immunity could delay infections so that more women acquire primary infection during pregnancy. This was also a concern during the development of rubella vaccine and is relevant because all vaccines increase the average

TABLE 13 Populations in whom efficacy of HCMV vaccines could be evaluated by using virologic endpoints

Population given vaccine/placebo	Outcome
Seronegative postpartum women[a]	Reduced primary HCMV infection
Seronegative workers at day care centers[a]	
Seronegative women with children at day care centers[a]	
Adolescents[a]	
Toddlers	
Seronegative patients on waiting list for receipt of solid organs	Reduced HCMV viremia/need for preemptive therapy
Seropositive patients on waiting list for receipt of solid organs	
Seropositive or seronegative recipients of stem cells	
Seronegative or seropositive donors of stem cells to seropositive recipients (adoptive transfer)	

[a]Females in this population who became pregnant may show a reduced rate of congenital transmission to their offspring.

age at which unvaccinated individuals acquire natural infection. Mathematical modeling shows that such a phenomenon will not produce a problem for HCMV vaccinees in a typical developed country because the average age of infection is already greater than the average age at which pregnancies occur (Fig. 14) (158). Modeling indicates that the basic reproductive number of HCMV in such a population is similar to that of smallpox, so by reducing the number of infectious individuals in a society, herd immunity could reduce the incidence of reinfections as well as primary infections (Fig. 15). Because reinfections are a more important cause of disease than reactivations in transplant patients (98) and probably in pregnant women as well (88,159), this prediction provides encouragement for the ultimate elimination of HCMV infection, although several generations of people would need to be immunized and eradication would take longer in communities where HCMV is acquired earlier in life. Nevertheless, we continue to hypothesize that control of initial HCMV infection could impair its ability to establish sanctuary sites protected by its immune evasion genes, so that individuals become less contagious to others. This optimistic scenario has already been proven true for other infections that persist in humans (hepatitis B virus, varicella-zoster virus, and human papillomavirus), so hopefully one or more of the current HCMV vaccine candidates will demonstrate the modest degree of protection required to allow this hypothesis to be tested.

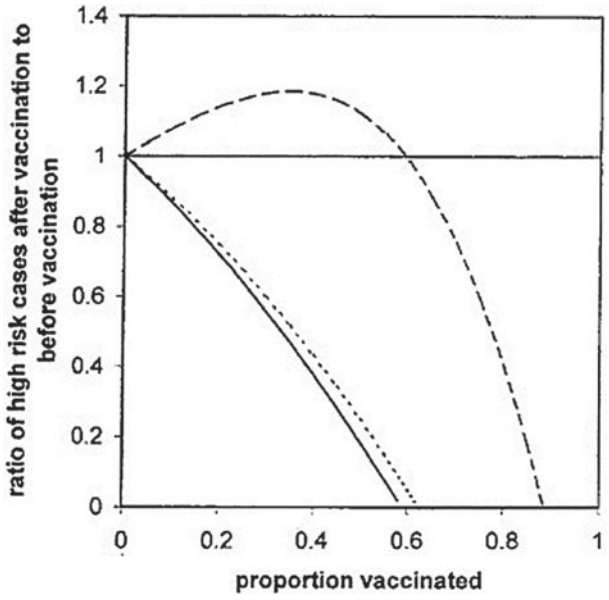

FIGURE 14 Herd immunity for HCMV or rubella ——, CMV study 1; ·····, CMV study 2; - - - - - -, rubella.

Passive immunoprophylaxis has been evaluated in three randomized controlled trials. Two were conducted in solid organ transplant patients, the largest of which reported significantly reduced CMV syndrome in liver transplant patients. The third trial was in pregnant women with primary HCMV infection in whom no significant reduction in intrauterine transmission of virus was seen, whereas an excess of complications of pregnancy was apparent (160). Thus, despite claims based on uncontrolled observations, hyperimmune immunoglobulin should not be offered to pregnant women in clinical practice.

TREATMENT

Transplant Patients

Before antiviral drugs became available, an important therapeutic decision in transplant patients with active HCMV infection was to reduce the dose of immunosuppressive drugs. This should remain a component of the management of HCMV infection, supported by the administration of specific antiviral drugs. In transplant patients, the availability of surveillance samples allows early treatment options in addition to those of prophylaxis and treatment of established disease. One key point is that lessons learned from one patient group cannot be applied directly to others.

Drugs with activity against HCMV could potentially be used in four main ways, depending in part on their toxicity profiles (Table 14). Sixteen double-blind, placebo-controlled, randomized trials conducted for HCMV are summarized in Table 15 according to four main criteria of success: prevention of HCMV infection, prevention of HCMV disease, reduction in mortality, and decreased indirect adverse effects (Table 15). In addition to ganciclovir and valganciclovir, several agents, including acyclovir, alpha interferon, and valacyclovir, have activity against HCMV *in vivo* (Table 15). In contrast to their relatively low potency when tested against HCMV *in vitro*, all of these treatments significantly reduced HCMV excretion, except immunoglobulin (Table 15). Quantitative virologic assessments were

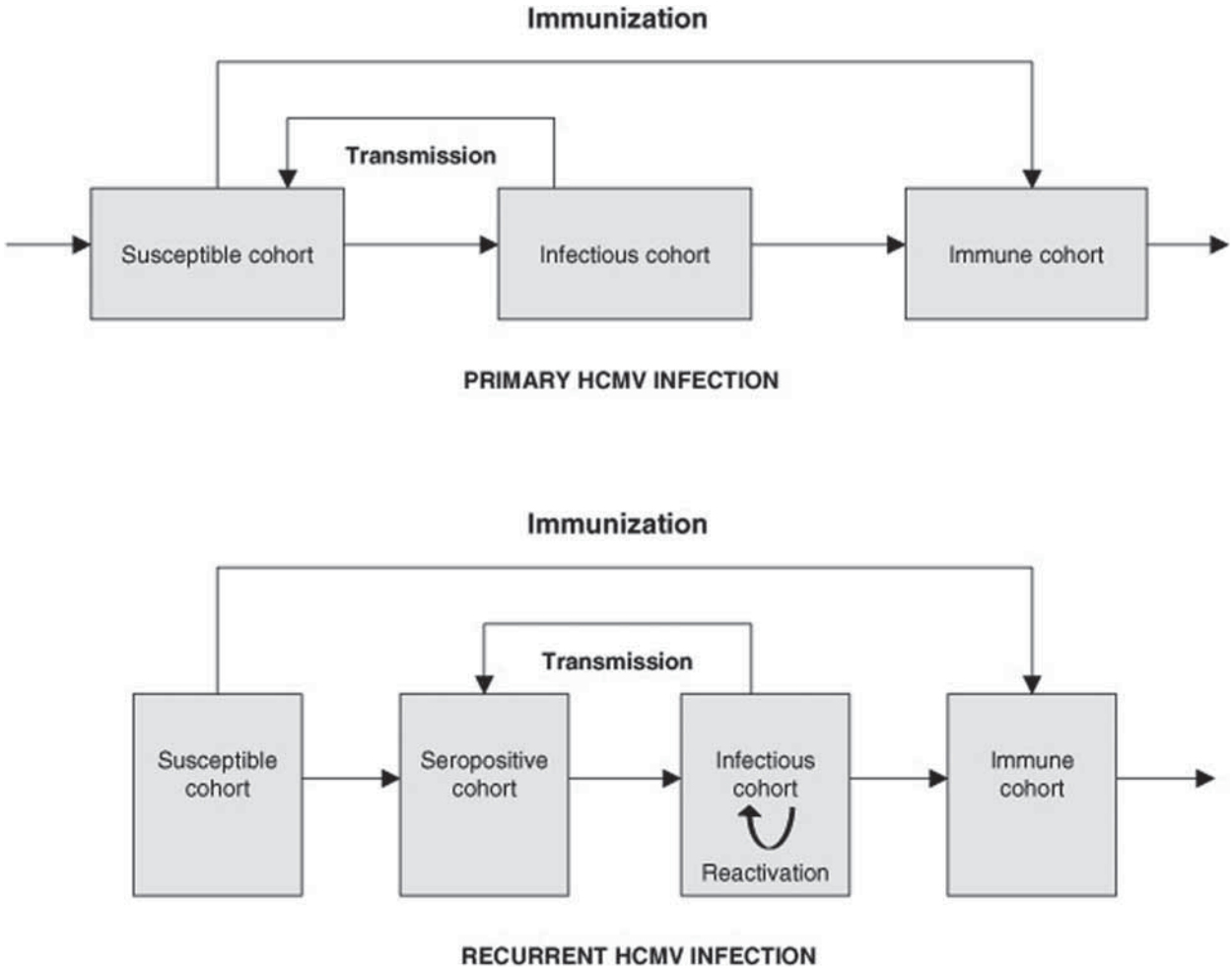

FIGURE 15 Standard population models of susceptible, infectious, and immune individuals, modified to include seropositive individuals who act as a source of HCMV for reinfections.

not done, but this suggests that any beneficial effect observed from the use of immunoglobulin is not necessarily mediated through a reduction in HCMV infection.

Only some of the studies have shown that control of HCMV infection leads to a reduction in HCMV disease (Table 15). The most consistent results come from the trials of ganciclovir, which clearly can markedly reduce HCMV disease in some groups (like stem-cell transplant patients) but inconsistently in others (an effect was seen in heart transplant patients for reactivation but not for primary HCMV infection in one study, whereas the converse was true in a second study). The apparent discrepancies among clinical trials (no significant effect for ganciclovir in one of two bone marrow transplant studies, an effect of acyclovir in renal transplant but not bone marrow transplant patients, and benefit in only two of the three alpha-interferon trials), can be explained by relatively small sample sizes. Each trial showed trends in favor of a reduction in HCMV disease.

When the parameter of clinical benefit was survival, only three studies showed a positive result (Table 15). Ganciclovir given to patients already excreting HCMV was lifesaving, but the same was not true in either of two studies that evaluated prophylactic ganciclovir in bone marrow transplant recipients. These studies showed that the bone marrow toxicity of ganciclovir, manifested as neutropenia, was associated with bacterial superinfection (94, 161). Because all patients in this prophylaxis study were exposed to potentially fatal side effects but only some benefited from a reduced mortality associated with HCMV, these two effects cancelled each other out. In contrast, when ganciclovir was used in the suppressive mode, a major benefit was achieved while exposure to drug toxicity was minimized. Acyclovir use in the same patient population provides an interesting contrast. Although this drug is less potent than ganciclovir, patients showed a survival benefit overall because there was no serious toxicity to offset its moderate efficacy (105).

When the parameter assessed is the ability to control the indirect effects of HCMV, both ganciclovir and valacyclovir show significant benefits. The original study by Merigan et al. (162) of heart transplant recipients utilized a regimen of intravenous ganciclovir suboptimal in terms of both dose and duration. Indeed, the trial reported that HCMV disease was reduced among seropositive but not seronegative recipients, who we now know have high viral loads. Nevertheless, this suboptimal regimen produced significantly reduced risks of fungal superinfections and accelerated atherosclerosis during long-term follow-up (163). In renal allograft patients randomly allocated valacyclovir, 2 g four times daily for 90 days, or matching placebo, seronegative recipients showed a 50% reduced risk of biopsy-proven acute

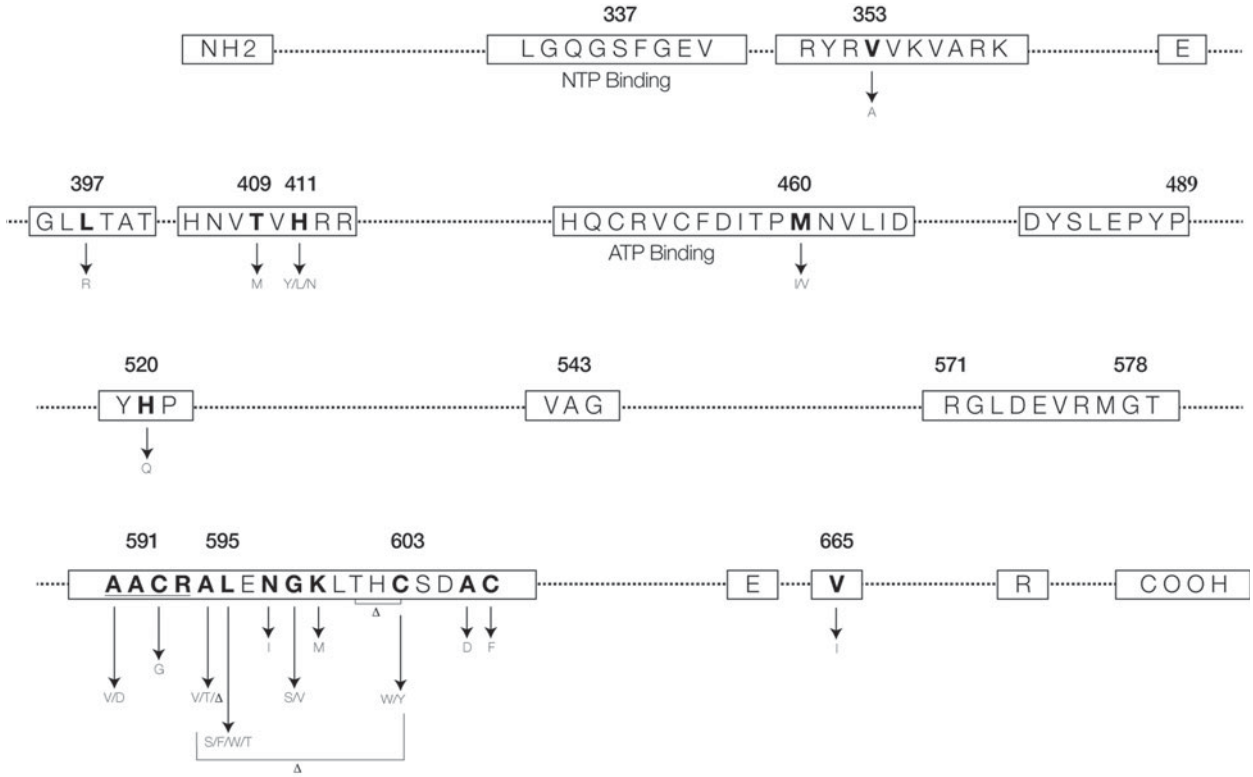

FIGURE 16 Schematic representation of the UL97 gene showing mutations at particular codons that have been proven to cause resistance to ganciclovir *in vivo* or that are associated with maribavir resistance *in vitro*. Bold letters indicate mutations confirmed by site-directed mutagenesis. Mutations 460 to 607 refer to ganciclovir; mutations 353 to 411 refer to maribavir. (Data from reference 189.)

graft rejections (133). Taken together, these results strongly implicate HCMV as a cause of the indirect effects listed in Table 7.

Meyers et al. (164) were the first to study acyclovir prophylaxis in bone marrow transplant patients. Among HCMV-seropositive patients, those who were also HSV seropositive and received high-dose intravenous acyclovir showed a marked reduction in HCMV disease and mortality rate. However, these encouraging results were not widely accepted, probably because at that time, acyclovir was not thought to have useful anti-HCMV activity. However, it is now known that the product of gene UL97 can phosphorylate acyclovir in addition to ganciclovir. A subsequent trial (105) based upon the protocol used by Meyers et al. (164) confirmed that acyclovir reduces the mortality in this patient group.

Among bone marrow transplant patients, Schmidt et al. (104) performed routine bronchoalveolar lavage on day 35 in asymptomatic patients and used the shell vial technique to detect HCMV infection. HCMV-infected patients randomized to receive ganciclovir had a marked reduction in HCMV pneumonitis following ganciclovir therapy, which contrasts with the failure of ganciclovir to improve survival once HCMV pneumonitis was established in these patients. This apparent contradiction is consistent with the hypothesis that HCMV pneumonitis is an immunopathological condition (102), presumably prevented by preemptive therapy (104). Bronchoalveolar lavage has now been replaced by routine detection of viremia as a less invasive way of identifying patients in need of preemptive therapy. Meanwhile, open studies of a combination of ganciclovir plus immunoglobulin suggest that better, but not excellent,

TABLE 14 Strategies for chemotherapy of HCMV

Term used	Time when drug is given	Risk of disease	Acceptable toxicity	Agent responsible for treatment decision
True prophylaxis	Before active infection	Low	None	Clinician
Delayed prophylaxis	When there is increased risk, but before active infection and after rejection	Medium	Low	Clinician
Suppression	After peripheral detection of virus	Medium	Low	Laboratory
Preemptive therapy	After systemic detection of virus	High	Medium	Laboratory
Treatment	Once disease is apparent	Established	High	Clinician and laboratory

TABLE 15 Double-blind, placebo-controlled, randomized trials for HCMV

Strategy	Drug	Reference to trial of drug use in: Bone marrow transplant	Renal transplant	Heart transplant	Liver transplant
Treatment	Ganciclovir	166[a]			
Preemptive therapy					
Suppressive Prophylaxis	Ganciclovir	104[a,b,c]			
	Acyclovir	164[a,c]	193[a,b]		
	Valacyclovir		133[a,b,d]		
	Immunoglobulin		194[c]		195[b]
	Ganciclovir	196[a]		162[a,b,d]	197[a,b]
		161[a,b]		198[a,b]	
	Maribavir	175[a]			
	Brincidofovir	191[a]			
	Letermovir	176[a]			
		190[a]			

[a]Reported significant benefit for HCMV infection.
[b]Reported significant benefit for HCMV disease.
[c]Reported significant benefit for survival.
[d]Reported significant benefit for indirect effects (including follow-up studies).

control of established HCMV pneumonitis can be achieved in this patient group (165, 166). Presumably, the immunoglobulin component has an immunomodulatory effect on HCMV immunopathological pneumonitis, whereas ganciclovir reduces the chance that neighboring lung cells will be recruited by HCMV to stimulate further immunopathology. It seems likely that an immunomodulatory effect of immunoglobulin would be nonspecific, and there is support for this from a rat experimental model (167). Thus, there is little evidence that immunoglobulin prepared from HCMV-positive donors has any advantage over immunoglobulin from unselected donors, but a comparative trial to test this formally would seem worthwhile. Foscarnet was equipotent with ganciclovir when used for preemptive therapy in bone marrow transplant patients (168). The combination of foscarnet plus ganciclovir (each at half dose) can be used to reduce toxicity without evidence of synergy (169).

In a controlled trial, low-dose intravenous ganciclovir prophylaxis was superior to high-dose oral acyclovir in liver transplant patients (170). In contrast to the case with bone marrow transplant patients, ganciclovir prophylaxis can be used safely in liver transplant patients, partly because of the lower dose chosen and partly because bone marrow toxicity may be a particular problem in bone marrow transplant patients.

Following the widespread introduction of antiviral prophylaxis in transplant patients, late-onset disease has emerged as a clinical problem that develops once patients stop their antiviral prophylaxis. Some patients develop disease due to ganciclovir-resistant mutants, and there have been serious cases of EOD, including death. Such late-onset disease is rarely seen in centers that use preemptive therapy (171). One plausible explanation is that low-level antigen presentation as part of preemptive therapy may stimulate the immune system to control long-term HCMV infection, analogous to "endogenous immunization." Alternatively, ganciclovir may interfere with the division of T cells, thus impairing their ability to form the most potent clonal derivatives. An important question for the field is whether prophylaxis per se selects for this immunologic problem or whether it is an adverse outcome of using ganciclovir prophylaxis. The results with newer drugs used for prophylaxis are therefore awaited with interest.

Both prophylaxis and preemptive therapy are effective strategies for preventing HCMV disease in transplant recipients. In addition, seven randomized clinical trials have directly compared prophylaxis and preemptive therapy in solid-organ transplant patients (172, 173, 176). A meta-analysis of these studies reported no significant differences in the incidence of HCMV disease, graft rejection, or death. Thus, while the relative merits of both strategies are hotly debated (171, 174), clinicians can use whichever strategy they find convenient for their transplant center.

Having managed patients for more than 20 years with ganciclovir plus occasional use of the other drugs mentioned, three new drugs have recently become available for clinical trials. They have all been evaluated in phase II studies, giving the drugs prophylactically to bone marrow transplant patients and recording whether the need for preemptive therapy with ganciclovir is reduced. All three placebo-controlled clinical trials were successful (175).

Maribavir is an inhibitor of UL97. Although strongly protein bound, sufficient drug can be administered to allow the free compound to inhibit HCMV replication. Maribavir is active against UL97 mutants and also against UL54 mutants resistant to ganciclovir, foscarnet, or cidofovir. However, because of its inhibition of UL97, maribavir results in a 13-fold increase in the 50% inhibitory concentration for ganciclovir, and consequently combination therapy with maribavir and ganciclovir will not be possible. The genetic changes in UL97 that confer resistance *in vitro* are summarized in figure 16.

Brincidofovir is a lipid prodrug of cidofovir. It lacks the renal toxicity of its parent compound and is distributed to all tissues in the body. It has significant activity against other viruses with double-stranded DNA, such as EBV, BK virus,

and adenoviruses. In a phase II study, prophylaxis with brincidofovir significantly reduced the need for preemptive therapy in stem cell transplant patients, but at the cost of dose-limiting gastrointestinal toxicity (176).

The CMV terminase complex cleaves concatemeric DNA into unit-length molecules as packaging proceeds by a "head-full" mechanism. Letermovir inhibits this complex and has activity against CMV only. In a phase II study, letermovir prophylaxis was more likely to prevent the need for preemptive therapy in stem cell transplant patients than was placebo (190).

All three of these new drugs have entered phase III clinical trials for prophylaxis in stem-cell transplant recipients. For the first (maribavir), the primary endpoint of CMV EOD was chosen, despite the fact that all patients were allowed preemptive therapy if maribavir or placebo failed to suppress viremia. In addition, a low dose of maribavir was chosen (191) In combination, these study design features led maribavir to fail its phase III evaluation (192). The lesson is clear; the effectiveness of prophylaxis should be determined by the ability of a drug to reduce the need for preemptive therapy.

None of the three new drugs has bone marrow toxicity. This means that if one or more of them become licensed, it could be given from the time of transplant onwards, as soon as patients can tolerate oral medication, rather than waiting for bone marrow engraftment to occur, as is the case with ganciclovir. The current delay in initiating antiviral prophylaxis with ganciclovir is undesirable, because better antiviral potency is obtained when treatment is begun before the patient has detectable levels of viremia.

AIDS Patients

In contrast to the logical approach to treatment described above for transplant patients, drug evaluation in HIV-positive patients has been largely empirical. Studies have focused on the clinical problem of established HCMV retinitis rather than targeting HCMV itself based upon knowledge of the natural history of infection.

The trial of the Studies of Ocular Complications of AIDS Research Group recruited patients with first-episode HCMV retinitis and randomly allocated them to receive either ganciclovir for induction and maintenance or foscarnet for induction and maintenance (177). Both were equally effective at delaying the time to recurrence of HCMV retinitis, but survival was significantly improved with foscarnet, despite the fact that treatment toxicity necessitating a switch to the alternate treatment was more common in those receiving foscarnet. The possible reason for the survival difference may relate to foscarnet's activity against HIV.

Two double-blind, controlled, randomized trials were conducted with HCMV-infected AIDS patients without EOD. These trials of oral ganciclovir (178) and oral valacyclovir (179) showed benefit in preventing disease. The strategy has been termed prophylaxis, but this refers to prophylaxis for disease and is not synonymous with prophylaxis of infection described above for transplant patients. Indeed, virologic assessment at entry shows that 50% of patients had HCMV infection detectable by PCR in urine, blood, or both. HCMV infection in HIV-positive patients occurs far earlier than has been appreciated by observing HCMV retinitis in AIDS patients. After 12 months, oral ganciclovir reduced HCMV retinitis from 30% in the placebo arm to 16%, whereas the valacyclovir trial reduced HCMV retinitis from 18% in the combined two-dose acyclovir arms to 12%. Ganciclovir showed significant benefit only in the subset who were PCR negative at baseline (i.e., true prophylaxis), whereas valacyclovir showed significant benefit for both preemptive therapy and prophylaxis (109, 180). This difference probably relates to the doses of the drugs administered rather than to their inherent potencies. Despite its known toxicity, ganciclovir was fairly well tolerated in these patients, partly because poor oral bioavailability may have limited the potential for toxicity. Valacyclovir at the high dosage chosen, 8 g/day, was poorly tolerated by AIDS patients, many of whom stopped therapy prematurely. Thus, the efficacy figures given above for the intention-to-treat analyses should be reviewed with the knowledge that many patients stopped treatment during the trial.

The results from these two trials are encouraging, but there remains much room for improvement. Drugs can now be targeted to reduce HCMV infection and decrease the chance of seeding the retina. One possible approach would be to monitor HIV-positive patients for evidence of HCMV infection by PCR and then start preemptive therapy. However, ACTG protocol A5030, in which patients whose plasma PCR was positive on a single occasion were given valganciclovir or placebo, found no significant benefit, but the study was seriously underpowered because of the difficulties of identifying individuals positive for HCMV as determined by PCR in the era of HAART (181).

The main strategy to prevent HCMV retinitis in AIDS patients is treatment with HAART regimens that maintain the CD4 count above 100. Indeed, patients who present with HCMV retinitis can have valganciclovir maintenance therapy stopped if their CD4 count rises above 100 and persists at that level for at least 3 months, showing that recovery of preexisting HCMV-specific immunity is sufficient to control progression of this EOD. In patients with lower CD4 counts, there is no consensus about whether to monitor for CMV viremia and what treatment to offer if CMV DNA is detected.

Neonates

A randomized trial in neonates with congenital HCMV and CNS symptoms or signs conducted by the Collaborative Antiviral Study Group (CASG), demonstrated significant control over progressive hearing loss from ganciclovir at 6 mg/kg twice daily given intravenously for 6 weeks (182). This benefit is consistent both with the increased viral load found in such neonates (90) and with the clinical observation that much of the hearing loss is acquired progressively after birth. Six weeks of treatment with intravenous ganciclovir became established as the standard of care for neonates born with CNS symptoms of congenital HCMV infection (182). Of note, ganciclovir has important toxicity (acute neutropenia and thrombocytopenia plus carcinogenicity in rodents at less than the human anticipated exposure), so evaluation of this drug should proceed with caution. Valganciclovir has been shown to be bioavailable orally in neonates (183), and a new CASG trial randomized congenitally infected neonates with symptoms at birth (not necessarily CNS symptoms) to receive 6 weeks versus 6 months of valganciclovir therapy (184). The longer duration of therapy provided significantly better control of hearing loss and effects on developmental milestones, although the incremental benefits seen were not as great as the fourfold increase in drug exposure, exactly as predicted by the threshold concept. This is now the new standard of care for babies born with symptoms affecting the CNS. Some pediatricians are reluctant to prescribe valganciclovir for 6

months if the symptoms at birth were mild, such as isolated thrombocytopenia, but no consensus has emerged on exactly which clinical criteria should guide treatment initiation.

Therapeutic Drug Monitoring

Therapeutic drug monitoring is only rarely required, especially when preemptive therapy is used and the patient's viral load can be monitored until it declines below the level of detection. If this expected pattern is not seen, there are two possibilities, poor absorption of drug or antiviral resistance. The first is usually addressed by giving ganciclovir intravenously, with declining viral loads often attributed to poor patient compliance with oral medication rather than poor absorption of valganciclovir. Therapeutic drug monitoring can be useful in occasional patients receiving hemofiltration whose viral load is static despite intravenous ganciclovir.

Resistance

Strains of HCMV resistant to ganciclovir have been found in AIDS patients receiving maintenance therapy for retinitis. Approximately 8% of urine samples contained resistant virus once patients had been treated for at least 3 months (185), which is probably an underestimate of the true situation *in vivo* because the long time taken to propagate HCMV in cell cultures allows the wild type to outcompete the resistant strain *in vitro* (111).

Most HCMV strains acquire resistance through mutations in the UL97 gene, although occasional mutations in DNA polymerase have been described. A range of mutations have been identified in UL54 that give rise to resistance to the antiviral drugs ganciclovir, foscarnet, and cidofovir. Some of these mutations, such as D301N, give rise to cross-resistance to all of the aforementioned nucleoside analogs. In a cohort of AIDS patients receiving oral ganciclovir maintenance therapy, 22% had resistance detected *in vivo* by genotyping (186, 187). None of the patients with resistance responded to reintroduction of ganciclovir, arguing that the laboratory results are clinically relevant. Knowledge of the rapid dynamics of HCMV replication *in vivo* has been used to explain and predict the development of resistance in AIDS patients (111). At present, resistance is not a major clinical problem because HCMV is controlled indirectly by HAART, but we anticipate further cases in the post-HAART era, and the principles discussed above should be reapplied. Specifically, patients who had genotypically resistant HCMV in the past may present again with HCMV disease poorly responsive to ganciclovir.

Increasing cases of ganciclovir resistance have been reported as antiviral prophylaxis has been used frequently in transplant patients. Mutations identical to those seen in AIDS patients develop. Late-onset HCMV disease, occurring after antiviral prophylaxis has been stopped, has emerged as a major clinical problem and is frequently caused by resistant strains. Alternative drugs to consider include foscarnet, letermovir, or brincidofovir, guided by the results of sequencing and the possibility of cross-resistance.

Novel Antiviral Targets

Fomivirsen is a licensed treatment for HCMV retinitis, although it is no longer marketed because of the diminishing number of cases of HCMV retinitis. It is an antisense oligonucleotide containing modified bases to reduce nuclease susceptibility. The drug interferes with the IE86 transactivator (Fig. 3) and has to be given by intravitreal injection.

The HCMV protease is an attractive target, especially since this class of inhibitor has been successfully developed for HIV. However, despite the availability of a three-dimensional structure since 1997, the search for inhibitors has been slow. This is partly compounded by the complexity of the enzyme and partly because the substrate binding groove is shallow, reducing the opportunities for small molecules to bind with high affinity.

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Human Herpesvirus 6 and Human Herpesvirus 7

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Human herpesvirus 6 (HHV-6) was first isolated from patients with lymphoproliferative disorders in 1986 and was initially named "human B-lymphotropic virus (HBLV)" (1). It was found to mainly infect and replicate in lymphocytes of T-cell lineage (2). Subsequently, several reports described the isolation of similar viruses mainly from patients with HIV/AIDS. The characterization of HHV-6 indicated that the virus was antigenically and genetically distinct from the other five known human herpesviruses (1, 3). HHV-6 isolates are classified into two closely related groups that have been named variants A (HHV-6A) and B (HHV-6B). Primary HHV-6B infection occurs during infancy. This virus was recognized as the causative agent of exanthem subitum (ES) in 1988 (4).

Another novel human herpesvirus, human herpesvirus 7 (HHV-7), was isolated in 1990 by Frenkel et al. from CD4+ lymphocytes of a healthy adult (5). Seroepidemiologic studies have indicated that the primary HHV-7 infection also occurs during childhood.

VIROLOGY

Classification

HHV-6A, 6B, and HHV-7 are members of the *Herpesviridae* family. Genomic analysis supports their classification as *Roseolovirus* genus of betaherpesviruses. On the basis of similarity between the amino acid sequences, gene organization, and putative protein functions, human cytomegalovirus (HCMV) is the closest phylogenetic relative of HHV-6A, 6B, and HHV-7 (6, 7).

HHV-6 strains, which were isolated in different regions of the world, are closely related to one another. Two distinct variants of HHV-6, named HHV-6 variant A (HHV-6A) and variant B (HHV-6B), exist, as demonstrated by restriction enzyme analysis, DNA sequence, and reactivity with monoclonal antibodies (MAb). Recently, these two variants have been classified as distinct species (8). Virus strains belonging to HHV-6A, including the original isolate (GS strain), are isolated mainly from patients with lymphoproliferative disorders or AIDS. HHV-6B strains are isolated mostly from patients with ES.

HHV-7 cross-hybridizes with some HHV-6A/B DNA probes. There are no recognized HHV-7 variants to date, but there is significant genetic variation among isolates. Predictably, there is also antigenic cross-reactivity between HHV-6A, 6B, and HHV-7 (6, 9).

Virus Composition

Virion Structure

Electron microscopic examination of infected cells showed that HHV-6 is an enveloped virion with an icosahedral capsid with 162 capsomeres (Figure 1) (10). Most of the capsids are in the nucleus and have cores of low density with a diameter of 90 to 110 nm. Enveloped viral particles are observed in cytoplasmic vacuoles as well as extracellularly, and have a diameter of 170 to 200 nm. In the nucleus, tubular structures were occasionally observed (11). Naked particles gradually acquire a full tegument in a nuclear vacuole. Fusion events with the nuclear membranes result in the release of the tegumented capsids into the cytoplasm. The tegumented capsids then undergo envelopment in cytoplasmic vacuoles, yielding mature virions. Fusion of the vacuole membrane with the cell membrane releases the intact virion into the extracellular space.

The virion glycoproteins are absent from nuclear and plasma membranes (12) but are concentrated in cytoplasmic structures known as *annulate lamellae* (13, 14). Final envelopment of viral particles occurs at intracellular membranes that are characteristics of endosomes and the trans Golgi network (15). HHV-6 virions collect in multivesicular bodies (MVB) that contain numerous mature virions and small exosomal vesicles (15). MVB membranes can fuse with the plasma membrane, resulting in release of mature virions. In addition to exocytosis, mature virions can be released by cell lysis (14).

Genomic and Genetic Properties

The HHV-6 genome is a linear, double-stranded DNA molecule with a size of 160 to 170 kbp composed of a central segment of a largely unique sequence (U) of approximately 141 kbp with a sequence of approximately 10 to 13 kbp duplicated in the same orientation at both left and right genomic termini (DR) (16). The DNA length variation map to the left end of both DR elements is termed the heterogeneous or het region (17, 18). The left and right termini of the DR contain homologs of the herpesvirus cleavage and packaging signals, pac-1 and pac-2, respectively. The mean G+C content of genomic DNA is 43% (17). The entire

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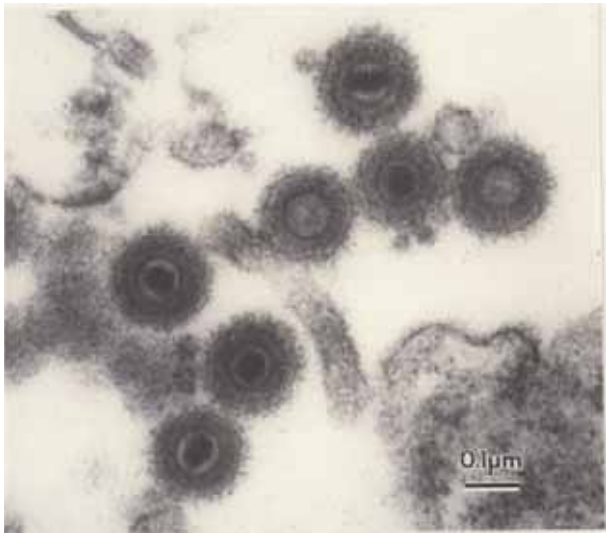

FIGURE 1 Herpes virus particles seen outside of HHV-6 infected cells.

HHV-6A (U1102 strain) (16), HHV-6A (GS strain) (19), HHV-6B HST (20), and Z29 (21) genomes have been sequenced. HHV-6A and HHV-6B have overall nucleotide sequence identity of 90% (Figure 2); the portions of the genomes that span U32 to U77 are highly conserved (95% identity), while the segment spanning U86 to U100 is only 72% identical (21).

HHV-7 also has a linear, double-stranded DNA of 140 to 160 kbp. The complete genomic DNAs of two strains (JI and RK) have been sequenced (22, 23). HHV-7 also possesses a similar genomic organization with a single long U flanked by identical direct repeats (DRL and DRR), yielding the arrangement DRL-U-DRR (24). Furthermore, there are pac-1 and pac-2 sequences and repeated sequences of GGGTTA at each end of DR motifs.

Over the conserved domains, *Roseoloviruses* are genetically co-linear with cytomegaloviruses, although the cytomegaloviruses encode many genes without roseolovirus counterparts. Within HHV-6A, HHV-6B, or HHV-7, these viruses exhibit relatively little genetic variability. Between HHV-6 and HHV-7, amino acid sequence identities range from 22 to 75%, most being in the vicinity of 50%. There is evidence for subgrouping within the HHV-6B at some genomic loci (25) and among HHV-7 isolates (26), but there is no indication that this is anything other than intraspecies allelic variation.

Protein Properties

More than 30 polypeptides with molecular masses ranging 30 kDa to 200 kDa, including 6 to 7 glycoproteins, are found in virions and HHV-6-infected cells (27–29). Glycoproteins gB and gH are essential for viral replication; induced neutralizing antibody inhibits the penetration of HHV-6 into susceptible human T-cell lymphocytes (30). HHV-6 gH/gL complex associates with glycoproteins Q1 and Q2 (gQ1 and gQ2) complex. The gQ, the products of the U100 gene, are unique to HHV-6 and HHV-7. The gQ2 interacts with the gH/gL/gQ1 complex in infected cells and virions to form gH/gL/gQ1/gQ2 complexes (31, 32). The complex is a viral ligand for its cellular receptor, CD46 (33). HHV-6 U47 is a positional homolog of the HCMV glycoprotein O (gO) gene (16), and also associates with gH/gL complex (32). gQ1 and gO have much greater sequence divergence than do other glycoproteins (76.8% and 72.1% identity, respectively), suggesting that they contribute to the biological differences. HHV-7 gO also associates with gH/gL complex in infected cells (34). HHV-6 and HHV-7 U21 encoded gly-

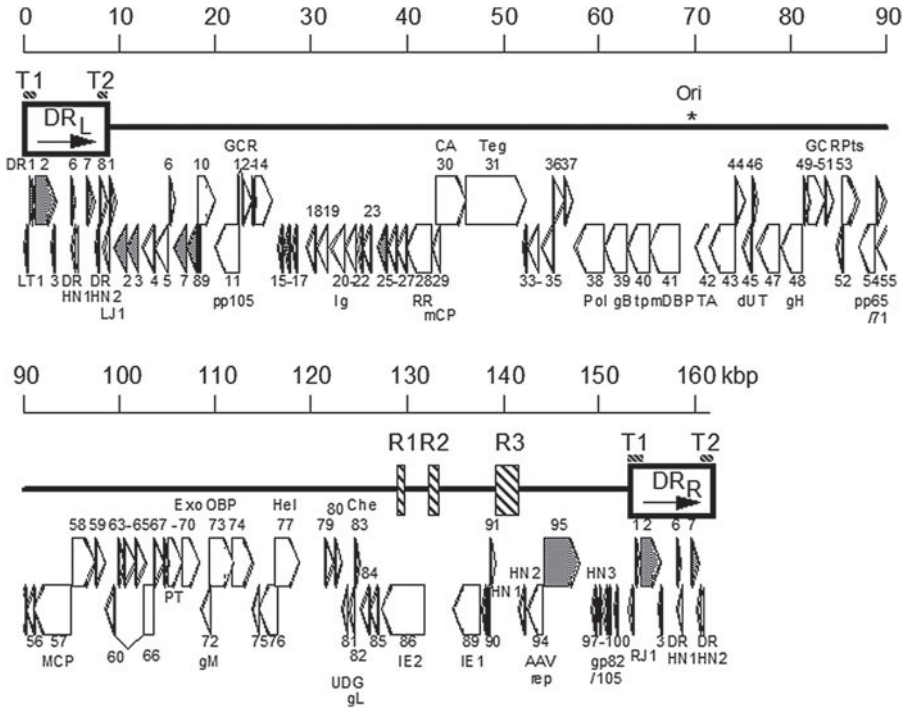

FIGURE 2 Genome structure of HHV-6B, HST strain; DR; direct repeat. (This figure was shown in a figure in reference 20.)

coproteins associate with major histocompatibility complex (MHC) class I molecules (35, 36), and the association functions in lysosome sorting, which may reduce cytotoxic T lymphocyte (CTL) recognition of infected cells. HHV-6 and HHV-7 U24 proteins induced internalization of the T-cell receptor/CD3 complex at the cell surface, resulting in abberant T-cell activation (37). HHV-6B IE1 interacts with STAT2 and sequesters it in the nucleus, preventing ISG3 from binding to IFN-responsive promoters, resulting in silencing of ISG (38).

The predominant protein species immunoreactive with human serum are p100 (HHV-6A) and p101 (HHV-6B) (39). These proteins function as a tegument protein like HCMV pp150. A polypeptide with an approximate molecular weight of 41 kDa is produced early in the replicative cycle (40). The immediate early proteins may transactivate the HIV long terminal repeat (LTR). HHV-6 also codes virus-specific DNA polymerase, uracil-DNA glycosidase and alkaline DNase, and induce thymidine kinase and deoxyuridine triphosphate nucleotidohydrolase. The HHV-6B rep gene, which is a homologue of adeno-associated virus type 2 rep and is unique in the herpesvirus family, has been identified (41). HHV-6 and HHV-7 encode viral chemokine and chemokine receptor homologs (42, 43). U83A encoded by HHV-6A binds a human chemokine, CCR5, and inhibits HIV replication *in vitro*, suggesting that HHV-6 U83A acts as a novel inhibitor of HHV-6 infection (44).

At least 20 proteins, including 7 glycoproteins, are specific to HHV-7 and range in apparent molecular weight from 136 to 30 kDa (45). Human antibodies raised to HHV-7 are directed predominantly to one or more HHV-7-infected cell proteins with apparent molecular weight of about 85 (phosphoprotein 85 or pp85) to 89 kDa. The pp85 is considered a major determinant of the human immune response to HHV-7, discriminating HHV-6 from HHV-7 infection. Further, human sera recognize additional epitopes of pp85 that are required for their full reactivity (46, 47). Some MAbs reacted in immunofluorescence assays with HHV-6 antigens to the same degree as to HHV-7 protein (45, 48).

Biology

HHV-6 is isolated only from human mononuclear cells (mainly CD4+ lymphocytes) and propagates in CD4+ lymphocytes (49, 50). HHV-6 infects a variety of human cells such as T and B lymphocytes of peripheral blood, cell lines of lymphocyte, and macrophage *in vitro*, and infects also lymphocytes of chimpanzees. The cytopathic effect (CPE) induced by HHV-6 begins 1 to 2 days post-infection as evidenced by enlargement of infected lymphocytes. These refractive giant cells usually contain 1 or 2 nuclei, and following the development CPE, lytic degeneration of the cells occurs. CD46 is a cellular receptor for HHV-6 (51). HHV-6A can induce fusion-from-without (fusion that does not require viral protein synthesis) in a variety of human cells, dependent on CD46 expression (52). Human CD134, which is expressed in activated T-cells, was found to be a cellular receptor specific for HHV-6B, but not HHV-6A (53). HHV-6B gH/gL/gQ1/gQ2 complex binds to CD134 and that of HHV-6A binds to human CD46 (53) (Figure 3).

HHV-6 has been shown to integrate into host cell chromosomes and be vertically transmitted in the germ line. HHV-6 specifically integrates into telomeres of chromosomes during latency rather than forming episomes, and the integrated viral genome is capable of producing virions (54). A single HHV-6 genome integrates such that telomere repeats join the left end of the integrated viral genome (55).

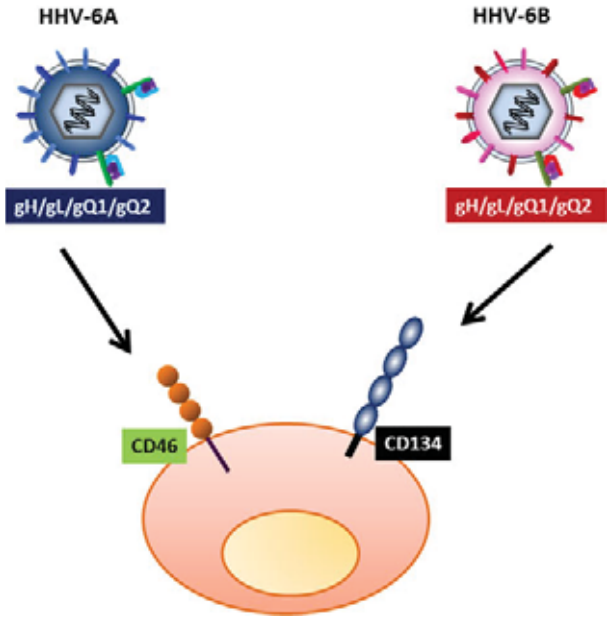

FIGURE 3 HHV-6A/B and cellular receptors. gH/gL/gQ1/gQ2 complex expresses in HHV-6A/B envelope. HHV-6A gH/gL/gQ1/gQ2 complex binds to human CD46, and that of HHV-6B binds to human CD134.

In contrast, HHV-7 uses CD4 as a receptor for infection in T-cells (56). Although all HHV-6 isolates can infect and replicate in human umbilical cord blood mononuclear cells, HHV-6A strains replicate in immature lymphocytes and more rapidly in a variety of established cell lines including EBV-positive B-cells, continuous T-cell lines, fibroblasts, megakaryocytes, and glioblastoma cells (2). HHV-6B strains fail to replicate in most continuous cell lines (57), with the exception of Molt 3 and MT-4 cells. The different cellular affinity between the two variants may be due to the lack of viral receptors for entry into the cells, or a deficiency in gene expression and viral replication. HHV-6B strains replicate in mature lymphocytes. Prior mitogen activation and full progression of the cell cycle are required for efficient replication, particularly for HHV-6B strains. High levels of IL-2 can delay or inhibit the viral replication (58).

Animal models of HHV-6 or 7 infections are lacking. Inoculation with the serum of a patient during the febrile period of ES transmitted a fever-producing agent to monkeys (59). Western blotting and imunofluorescent antibody assay (IFA) demonstrated the presence of HHV-6 antibody in monkeys (60). Chimpanzees are susceptible to productive infection by HHV-6 (61).

EPIDEMIOLOGY

Seroprevalence

The prevalence of antibody to HHV-6B is high in most, but not all, populations of the world. An HHV-6B serological study in Japan found that the incidence of antibody in different age groups was similar, even in those younger than 10 years. From 6 months of age, the number of children having antibodies gradually increased, and almost all children older than 13 months were seropositive, indicating past infection (62). This indicates that almost all children are exposed to HHV-6B in the latter half of the first year of life. Since most

pregnant women have antibody to HHV-6B, immunoglobulin G (IgG) antibody is transferred from mother to child across the placenta, and is detectable in infants during the early months after birth. Very young infants (younger than 6 months) are probably protected against HHV-6B infection by antibodies from their mother.

HHV-6A seems to predominantly infect infants in sub-Saharan Africa (63), and both HHV-6A and B infect persistently in the same individuals (64).

Infection by HHV-7 occurs slightly later in life than HHV-6 (9, 65), although a study in the United Kingdom indicated no difference in the prevalence of antibody to both viruses with respect to age-matched controls (66). No antibody was detectable in children younger than 2 years in the United States (9), but the seropositivity to HHV-7 was 75% in children aged 12 to 23 months in Japan (67).

Mode of Transmission

The mode of transmission of HHV-6 and -7 to children is not fully understood. No difference has been found in the prevalence rates of HHV-6B infection between breast- and bottle-fed infants (68) or between babies born by cesarean section and those born vaginally. HHV-6B DNA has been detected in saliva and throat swabs of ES patients by polymerase chain reaction (PCR), as well as in healthy adults including mothers (69–71). HHV-6B is also present in vaginal secretions of pregnant women (72, 73). HHV-6 reactivation seems common during pregnancy, and HHV-6B infection of the fetus may occur.

Local spread and seasonal outbreaks of ES are rare, although outbreaks of HHV-6B infection are occasionally observed among institutionalized children (74). Persistent excretion or recurrent episodes of shedding HHV-6B from saliva and cervical secretions of adults suggest that close contact is a mode of transmission early in life, mainly from mother to child. Specific DNA could be detected in 1 to 1.6% of cord blood specimens from babies born to ostensibly healthy mothers (75, 76). Since an intact HHV-6A or B genome is universally integrated in several distinct chromosomes (77, 78), congenital transmission of HHV-6 can be caused by either genetic transmission of chromosomal integration of HHV-6 (ciHHV-6) from mother or father or both parents, or by transplacental transmission with ciHHV-6 (79, 80).

HHV-7 can be frequently isolated from saliva of the healthy adults (81–83), and horizontal transmission of HHV-7 may occur even from grandparents or parents to children through close household contact. Thus, the transmissions of HHV-6 and HHV-7 are very similar; however, it is not clear why HHV-7 infection occurs generally later than HHV-6 infection. There is no report of ciHHV-7 to date.

PATHOGENESIS

The mechanisms by which HHV-6 induces pathology in humans have not been precisely defined. Suppression of bone marrow function is one of the most serious effects of HHV-6 infection in bone marrow transplant recipients. Immunological and molecular analyses indicate that CD4+ T lymphocytes are the predominant target cells for HHV-6. Direct viral cytolysis may be responsible from acute diseases such as ES and heterophile-negative infectious mononucleosis. Besides the direct infection of immune cells, indirect pathogenic mechanisms following HHV-6 infection may result from modulation of the immune system. Infection of PBMC by HHV-6A suppressed IL-2 synthesis *in vitro*, and levels of IL-2 transcripts were diminished (84). Recently virus specific regulatory T-cells were induced by HHV-6, suggesting immunosuppression in infected hosts (85).

HHV-6 up-regulates CD4 (86) and NK cells (87) and down-regulates CD3 molecules in T-cells (88). It also induces the release of IFN-α (89–91), IL-1b, and tumor necrosis factor alpha by infected cells (92). Such altered polyclonal cell stimulation and cytokine bursts might contribute to the development of lymphoproliferative disorders, including lymphoma and leukemia.

These proinflammatory cytokines can upregulate HIV replication *in vitro* and contribute to the pathogenesis of AIDS. Both viruses infect CD4+ T lymphocytes. HHV-6 expression transactivates HIV-1 LTR. HHV-6 infection also induces CD4 molecules on the surface of CD4-CD8+ cells at the transcriptional level, resulting in enhanced susceptibility to HIV-1 infection (86, 93, 94). This concept remains controversial since other reports suggest inhibition of HIV replication in the case of co-infection (3, 95–97).

HHV-6A dramatically accelerates progression from HIV to full blown AIDS in monkeys (98). Since HHV-6 is neuroinvasive, as described below, it may contribute to the neuropathogenesis of HIV/AIDS. HHV-6 is extensively disseminated in neural cells in brains of HIV-infected patients, suggesting a contribution to the pathogenesis of AIDS encephalopathy (99). In contrast, HHV-7 down-regulates CD4, the cellular receptor shared by HIV and HHV-7. HHV-7 suppresses the replication of CCR5 tropic HIV isolates through CD4 modulation, suggesting that HHV-7 and HIV-1 may interfere in lymphoid tissue *in vivo* (100).

As described above, HHV-6 and HHV-7 establish latency after primary infection, but the mechanism is not understood completely. An immediate-early 1 protein of HHV-6 inhibits transcription of the IFN-β gene and may contribute to latency (101).

CLINICAL MANIFESTATIONS

Acute Infection

Primary HHV-6 infection in early infancy causes ES (roseola infantum), a common illness characterized by high fever for a few days and the appearance of a rash coinciding with defervescence. The rash appears on the trunk and face and

FIGURE 4 Clinical feature (typical rash) of a patient with exanthem subitum.

spreads to lower extremities during subsiding fever (Figure 4). Human-to-human transmission has been documented by experimental inoculation of blood from ES patients into other infants (59, 102). Novel cell culture techniques allowed isolation of the virus (4). HHV-6 infection can occur without clinical symptoms of rash or fever (68, 103–106). When people escape childhood HHV-6 infection and are infected as adults, they develop a self-limited febrile disease that resembles infectious mononucleosis (47, 107).

The clinical features of primary HHV-7 infection have not been established. In 1994, HHV-7 was isolated from peripheral blood mononuclear cells of two infants with typical ES and the DNA patterns of the isolated viruses which were digested with various restriction enzymes very similar to that of the prototype HHV-7 (RK strain) (108). During the convalescent period of one patient, the antibody titer to HHV-7 increased significantly whereas the antibody titer to HHV-6 remained negative. In the second patient, who had two independent episodes of ES over 2 months, both HHV-6 and HHV-7 were sequentially isolated; HHV-6 seroconversion occurred during the first episode and HHV-7 seroconversion occurred during the second episode. These results suggest that HHV-7 is also a causative agent of ES. Temporally, isolation of HHV-7 before HHV-6 is uncommon; thus, it is still not completely clear whether HHV-7 causes ES-like symptoms.

ES is a common disease of infancy, and the symptoms are usually mild. Normally, children can recover from this disease after a few days without any complications. However, a few infants show hepatic dysfunction associated with ES (109, 110). Another common complication associated with ES is seizures (111), reported to occur in 0.6 to 50% of patients, but the exact incidence of seizures occurring with ES is difficult to estimate. Thus, ES has been suggested to be a risk factor for recurrent febrile seizures. Encephalitis and other complications of the central nervous system (CNS) have been also reported (112–114). HHV-6 DNA can be detectable in the cerebrospinal fluid of some of patients (115). These data suggest that HHV-6 may invade the brain during the acute phase of ES. HHV-6 has also been detected by PCR in brains of normal cadavers and brains of AIDS patients (116). HHV-6 may also cause lymphadenitis (117, 118). Idiopathic thrombocytopenic purpura has been associated with primary infection of HHV-6 (118).

Primary HHV-7 infection results in a febrile illness in childhood, complicated by seizures (119), and children with ES developed CNS disease including acute hemiplegia (120), suggesting that HHV-7 may also infect the brain. One study in hospitalized children during the first two years of life (121) found that 17% of the encephalitis cases were associated with primary infections of HHV-6 and 7, and that the two viruses contributed equally.

Diseases During HHV-6 and HHV-7 Reactivation

After primary infection, all three viruses establish latency and can be reactivated to cause disease, particularly immunosuppressed states. The site of latent infection with HHV-6 is unknown, but HHV-6 antigen has been detected in salivary glands. HHV-6 DNA can be detected in peripheral blood of normal adults at low frequency, as well as in both the monocyte/macrophages and lymphocytes during the acute phase of ES and mainly in monocyte/macrophages of patients in the convalescent phase and of healthy adults (121).

Reactivation of HHV-6 occurs after bone marrow transplantation (BMT), solid-organ transplantation, and AIDS. Asymptomatic HHV-6 reactivation is common in allogeneic BMT patients (122); symptomatic HHV-6 reactivation may result in bone marrow suppression (90), encephalitis (123), pneumonitis (124), and acute graft-versus-host disease (125). In fact, HHV-6 DNA is detectable in cerebrospinal fluid of patients after BMT and stem-cell transplant (126).

An association between rejection of transplanted kidneys and HHV-6 reactivation has been reported (127). HHV-6 reactivation in liver transplant recipients is associated with severe cytopenia (128, 129). Liver transplant recipients with ciHHV-6 experience more bacterial infections and allograft rejection than those without ciHHV-6 (130). Other associations include interstitial pneumonitis due to HHV-6, life-threatening thrombocytopenia, progressive encephalopathy, and rash in adults. HHV-6, HHV-7, and HCMV may contribute to the disease burden by reactivating at the same time during the course of transplantation (131).

HHV-6 has been demonstrated in some neoplasms, including non-Hodgkin's lymphoma and Hodgkin's disease. A high prevalence of HHV-6 has been found in oral, particularly salivary gland, carcinomas (132). A possible association of HHV-6 with the unusual disorder S100-positive, T-cell chronic lymphoproliferative disease has been reported (133). The frequency of HHV-6 variants in certain tumors suggests that they might serve as cofactors in multistep carcinogenesis, but there has been no conclusive demonstration that HHV-6 plays a causative role in any malignancy.

A reported association between chronic fatigue syndrome (CFS) and HHV-6 infection (134, 135) requires confirmation. More CFS patients than controls had elevated levels of HHV-6 early-antigen specific IgM, perhaps indicating active replication of HHV-6 in CFS (136). Further work is required to determine whether HHV-6 contributes to the clinical manifestations.

Several studies have suggested an association between HHV-6 and multiple sclerosis (MS). A DNA fragment containing the major DNA binding protein gene of HHV-6 was detected in the brains of patients with MS by representational difference analysis, as reviewed (137). Examination of 86 brain specimens by PCR demonstrated that HHV-6B was present in the brains of >70% of both MS patients and controls. Nuclear staining of oligodendrocytes using MAb against HHV-6 virion protein 101K and DNA binding protein p41 has been observed in samples from MS patients but not in controls. Samples from MS patients showed prominent cytoplasmic staining of neurons in gray matter adjacent to plaques, although neurons expressing HHV-6 were also found in certain controls (138, 139). One study reported increased IgM serum antibody responses to HHV-6 early antigen (p41/38) in patients with relapsing-remitting MS, compared with those with chronic progressive MS or other patients with neurologic disease, autoimmune disease, and normal controls. Other groups, however, have not confirmed these findings (140) and further studies are required (141).

HHV-6 DNA has been detected in brain tissues from patients with mesial temporal lobe epilepsy (142, 143); this association was restricted to patients with history of encephalitis (144). Numerous studies have found that HHV-6B persistently infects the human brain.

Detection of HHV-6 DNA has been reported in affected heart tissues from children and adults with acute myocarditis (145, 146).

Drug-induced hypersensitivity syndrome is characterized by a severe, potentially fatal, multi-organ reaction that usually appears after prolonged exposure to certain drugs (147). Clinical features overlap those for Stevens-Johnson

syndrome and toxic epidermal necrolysis. Clinical signs include a maculopapular rash progressing to exfoliative erythroderma, fever, and lymphadenopathy. It is uncertain whether the virus reactivation causes the disease, is triggered by the disease, or contributes to the disease.

Association of pityriasis with HHV-7 infection is a clinical presentation of HHV-7 reactivation (148). No significant differences in DNA and antibody tests are noted between patients and control groups. Further experiments are necessary.

LABORATORY DIAGNOSIS

Virus Isolation and Assay

HHV-6B is easily recovered from the peripheral blood lymphocytes of ES patients on the first or second day of the disease, which is during the febrile phase of ES, but the isolation rate gradually decreases thereafter. Virus has been isolated on some occasions from saliva, but the isolation rate is extremely low.

An effective method for virus isolation (4) involves use of uninfected cord blood lymphocytes. Cord blood cells are recommended because HHV-6 and HHV-7 may latently infect and reactivate during cultivation in peripheral blood mononuclear cells. CPE, with characteristic, balloon-like syncytia, usually appears 2 to 4 days post-infection. Mitogen-stimulated human cord blood mononuclear cells and adult peripheral mononuclear cells are readily infected by HHV-6. HHV-6A also infects HSB-2, an immature T-cell line, and HHV-6B infects several T-cell lines, including MT-4 and Molt 3 cells. Since high concentrations of IL-2 inhibit viral replication (58), culture medium after infection should contain only very low concentrations of IL-2.

HHV-7 is occasionally isolated from peripheral blood of patients with ES, and frequently from saliva of individuals who have antibody to HHV-7. Inoculation of salivary samples onto mitogen stimulated human cord blood lymphocytes results in CPE by 2 to 4 weeks.

DNA Detection

HHV-6 DNA can be detected by DNA hybridization and by PCR. Southern blot hybridization is useful for rapid screening of large numbers of specimens, but it is generally a less sensitive technique than PCR. Numerous PCR primer sets, which are sensitive and specific, have been described for HHV-6 DNA, and some of these allow easy discrimination of the variants (25, 149 – 151). Variant-specific oligonucleotide hybridization is based on the amplification of two distinct regions of the HHV-6 genome, followed by hybridization of amplicons with variant-specific oligonucleotide probes. The putative coding region of immediate-early genes of HHV-6A is 2,517 nucleotides long, and two large additional regions of 108 and 228 bp were found in HHV-6B. PCR ampflication, using primers covering one of these regions results in PCR-products with different molecular mass, permitting discrimination between HHV-6A and 6B. HHV-6 DNA is easily detectable by PCR in peripheral blood of ES patients during the acute phase, but detection of cell-free virus in serum or plasma by PCR offers the possibility of diagnosing active HHV-6 infections.

A RT-PCR assay can determine the presence of HHV-6 RNA in clinical specimens. The primers for amplification of mRNA of a major structural gene (gQ), which has a spliced structure and is expressed as a late gene and its mRNA, are employed. Therefore, the amplification of this gene has the advantage of detecting replicating virus and readily distinguishes mRNA from residual DNA contamination. This method showed a low false-positive rate (1.2%) and a high specificity of 98.8%.

A quantitative competitive PCR assay for HHV-6 has demonstrated the persistence of a high HHV-6 load in the absence of apparent disease (152). The primer sequences based on consensus sequences in the DNA polymerase gene of herpesviruses can be used for testing for six different herpesviruses simultaneously (153).

PCR can also be used for detection of HHV-7 DNA in a throat swab or peripheral blood. The method is essentially the same as for the detection of HHV-6 DNA. Qualitative and competitive-quantitative nested PCR assays have also been developed for the detection of HHV-7 DNA. These assays amplify a DNA sequence encoding part of the HHV-7 U42 gene (154).

Loop-mediated isothermal amplification (LAMP) is a novel technique of rapid detection of HHV-6 DNA using simple and relatively inexpensive equipment, making it suitable for rapid diagnosis of roseola (80, 155).

A multiplex PCR method was also developed for the simultaneous detection of HHV-6 and HHV-7 in clinical samples, using primers to amplify a segment of the HHV-6, U67 gene, and the HHV-7, U42 gene. Comparison of the multiplex assay with the respective single PCR assays, using cloned HHV-6 and HHV-7 sequences as targets for amplification, demonstrated equivalent sensitivity and specificity of the assays. This multiplex assay is an efficient and cost-effective approach to the analysis of large numbers of samples to determine the epidemiological importance of HHV-6 and HHV-7(156).

Since both HHV-6A and B have been found integrated into the chromosomes of immunocompetent patients at persistently high levels of viral DNA in blood, sera, and hair follicles (157, 158,159) (Figure 5), the PCR test should be carefully interpreted for diagnosis, even in cerebrospinal fluid.

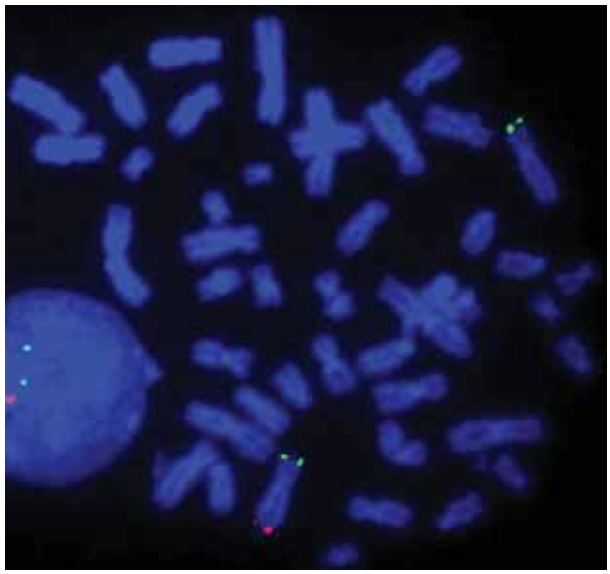

FIGURE 5 Fluorescent *in situ* hybridization (FISH) on metaphase chromosomes from a patient shows integration of HHV-6. The red and green signals indicate HHV-6 DNA and chromosome 6, respectively (the method is written in reference 159.)

Antigen Detection

A quantitative antigenemia assay using monoclonal antibody enables enumeration of HHV-6B-infected cells and has been used to monitor HHV-6 activity in liver transplant recipients (160).

Latent and replicating virus can be discriminated by assays that combine reverse transcription and PCR (RT-PCR) (144, 161, 162). Such assays target spliced mRNAs, enabling easy discrimination between mRNA and residual DNA contamination.

Serological Assays

Several serological assays are available for HHV-6 studies, including IFA, enzyme-linked immunosorbent assay (ELISA), neutralization (NT), radio-immunoprecipitation, and immunoblotting. Indirect IFA is presently the most commonly applied method for HHV-6 viral antigen and antibody detection. Separation of serum IgM from IgG and IgA significantly increases the specificity of HHV-6-specific IgM detection. Density gradient columns and protein A absorption and anti-IgG treatment techniques can be applied for removal of interfering substances such as heterotopic cross-reactions, rheumatoid factors, and antinuclear antibodies.

NT antibody tests using a CPE reading, chemically attached MT-4 cells (T-cell line), or dot blot assays have been reported (163). Neutralizing antibody titers appear to correlate with the antibody titers determined by the indirect IFA test (164).

An enzyme immunoassay, an immunoblot assay, and an indirect IFA have been developed for the detection of HHV-7 antibodies in human serum. Cross-absorption studies with ELISA or IFA using HHV-7 and HHV-6 antigens indicated that most human sera contain cross-reactive HHV-6 and HHV-7 antibodies. The degree of cross-reactivity varies between individual serum specimens. An 85/89 kDa protein was identified as an HHV-7-specific serologic marker by immunoblot analysis (165). To detect antibody to HHV-7 specifically and sensitively, the p89/85 proteins were expressed as recombinant proteins in bacteria, and developed for immunoblot kit. Of the three assays, the ELISA is the most sensitive, while the immunoblot assay is the most specific (166). A recently described assay appears to enable HHV-6A and B-specific serologic testing (167).

From a natural history perspective, IgM antibodies are detected by day 7 and persist for 3 weeks, but are not detectable in most sera 1 month after the onset of disease. IgG antibody is first detected 7 days after the onset of illness, increases in titer until 3 weeks after onset, and persists at least 2 months. Interesting, antibody titers to HHV-6 are boosted during other virus infections such as HHV-7(62, 145) or measles (168).

TREATMENT AND PREVENTION

No effective measures have been determined for the prevention of infection. Similarly, no vaccines are in development.

Many anti-HHV-6 and HHV-7 drugs have been described, but three drugs which have been developed for HCMV treatment can be used for HHV-6 and HHV-7 infections, as reviewed in (169). Ganciclovir, cidofovir, and foscarnet are inhibitors of DNA polymerase of these viruses. Other nucleoside or nucleotide analogues are efficient *in vitro* and are under preclinical stage or early clinical phase (169, 170).The sensitivity of HHV-7 to the guanine analogs differed from HHV-6, suggesting a different selectivity of specific viral enzymes (171). Prophylaxis with ACV did not prevent the occurrence of HHV-6-associated CNS disease after allogeneic bone marrow transplantation. IFN-α and IFN-ß inhibit HHV-6 replication *in vitro* (91).

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Epstein-Barr Virus

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25

In the century preceding the discovery of Epstein-Barr virus (EBV), physicians speculated that a common clinical syndrome characterized by fever, tonsillar adenopathy, splenomegaly, and mononuclear leukocytosis termed glandular fever was caused by a pathogen (1). In 1920, the name infectious mononucleosis (IM) was introduced by Thomas P. Sprunt and Frank A. Evans to characterize this syndrome (2). Three years later, Hal Downey and C.A. McKinlay described the now-classic atypical lymphocyte as a common feature of this disease (3), and in 1932 John Rodman Paul and Walls Willard Bunnell demonstrated high titers of spontaneously occurring heterophile antibodies in the sera of patients with IM (4), ensuring more accurate diagnosis. In 1961, the British surgeon Denis P. Burkitt gave the first account outside of Africa of "The Commonest Children's Cancer in Tropical Africa" at Middlesex Hospital London, detailing the geographic relationship between Burkitt's lymphoma (BL) and conditions of temperature, altitude, and rainfall associated with development of *Plasmodium falciparum* malaria (5, 6). M. Anthony Epstein, who was in the audience, became intrigued by the idea that a biological agent might be involved in the etiology of BL, and in 1964, the Epstein laboratory analyzed BL biopsy samples by thin-section electron microscopy and discovered a new, large, icosahedral herpesvirus that could be directly reactivated from *in vitro*–grown BL cells (7). These initial findings were reported in *The Lancet*, and the virus was named after Epstein and his graduate student Yvonne Barr (8). Shortly thereafter, two independent groups (9, 10) demonstrated the ability of EBV to transform primary human B lymphocytes into permanently growing lymphoblastoid cell lines (LCLs), providing the first concrete evidence of the ability of EBV to promote human cancer. In 1968, Gertrude Henle and Werner Henle made two further critical observations: (1) that EBV seroconversion occurred during the course of acute IM (AIM) in a laboratory technician (9) and (2) that EBV-carrying LCLs spontaneously formed from a peripheral blood leukocyte culture obtained during the acute phase of the technician's illness. The Henles confirmed their observations through study of sera provided by James Corson Niederman and Robert W. McCollum, who collected blood from incoming Yale freshmen and later from individuals who developed AIM. This study and others demonstrated EBV-specific antibodies in the sera of the students who developed AIM, confirming the etiologic association between EBV and AIM (11).

VIROLOGY

Classification

EBV is a member of the Gammaherpesvirinae subfamily of the family Herpesviridae and is the prototype of the *Lymphocryptovirus* genus (gamma-1 herpesvirus). *In vitro*, all gamma herpesviruses infect and replicate in lymphoid cells and variably in other hematopoietic lineage cells, epithelial cells, smooth muscle cells, and fibroblasts. The host range of the *Lymphocryptovirus* genus is restricted to primates. *Lymphocryptovirus* infection of primate B lymphocytes results in persistence of the viral genome, which is maintained through variable expression of a restricted set of latent gene products that typically sustain quiescence, but can also help drive cell proliferation and contribute to the transformation process (12).

Type and Strain Variations

The quest to define what constitutes "wild-type" EBV for vaccine development and to determine whether genetic strain differences correlate with specific EBV-associated disease began shortly after sequencing of the entire EBV genome in 1984 (13, 14). Two main types of EBV, namely EBV-1 and EBV-2 (or A and B, respectively), were identified in distinct global populations on the basis of analysis of the strains then available (15). With the advent of advanced DNA sequencing technologies, there is now sequence information from at least 83 independent EBV strains originating from different parts of the world, and much work is ongoing. Identification of additional polymorphisms has added to the complexity of interpreting whether strain variants predict disease. Although most EBV genes differ in sequence by <1% to 5%, the EBV-1 and EBV-2 latent infection cycle nuclear antigen gene 2 (EBV nuclear antigen 2 [EBNA-2])-encoded proteins share only 54% identity. African EBV genomes are almost as frequently type 2 as type 1, in contrast to American, European, and Asian EBV genomes, which are 10 times more likely to be type 1 than type 2.

Individuals, particularly those who are immunocompromised, can be infected with multiple strains, and new polymorphisms can arise within an infected host. Type 1 EBV

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immortalizes B lymphocytes *in vitro* with greater efficiency than type 2, which is reflected by the more rapid outgrowth of LCLs, growth to higher saturation density, and increased frequency of tumor formation upon inoculation of severe combined immunodeficient (SCID) mice (16). Although the molecular mechanisms underlying these *in vitro* differences are understood increasingly (17), there remains little *in vivo* evidence that one type is more likely than another to cause any EBV-associated disease. Strain polymorphisms with potential disease associations in gene products, such as latent membrane protein 1 (LMP-1, a viral oncoprotein) (18) in nasopharyngeal carcinoma, the EBV nuclear antigen 3B (EBNA-3B) gene (an ostensible tumor suppressor) in B-cell lymphomas (19), and several others, have been described (20, 21). However, substantive evidence of broad disease associations is lacking. Future comprehensive DNA analyses that incorporate sequences of healthy controls from diverse geographic regions should provide important new information (17, 22, 23) about the clinical impact of polymorphisms.

Virion Composition and Genome Structure

The EBV virion is composed of linear double-stranded DNA that is devoid of histones. The DNA is packed into an icosahedral nucleocapsid containing 162 capsomeres and surrounded by an amorphous tegument formed by protein and RNAs. Surrounding this is an outer viral envelope, which consists predominantly of alternatively spliced glycoproteins known as gp350/220 (hereafter referred to as gp350) (12) (Fig. 1). Intact virions contain ~35 EBV proteins as well as 5 cellular proteins (24).

The EBV genome was initially characterized in 1970 and was completely sequenced in the early 1980s by the Sanger method using overlapping BamHI and EcoRI restriction enzyme fragments (14). Individual EBV gene products were named on the basis of the BamHI restriction fragment in which transcription was initiated (e.g., Bam A, B, . . . Z). Included in this nomenclature was whether RNAs were transcribed from a rightward open reading frame (RF) or a leftward open reading frame (LF) relative to the site of origin. Characteristic features of the EBV genome, as depicted in Fig. 1, include a single overall gene arrangement, tandemly reiterated 0.5-kbp terminal repeats, and tandemly reiterated 3-kbp internal repeats (W repeats) that divide the genome into unique long and short regions. EBV DNA is linear in the virus particles, but it rapidly circularizes through the terminal repeat sequences in infected cells. Each infected

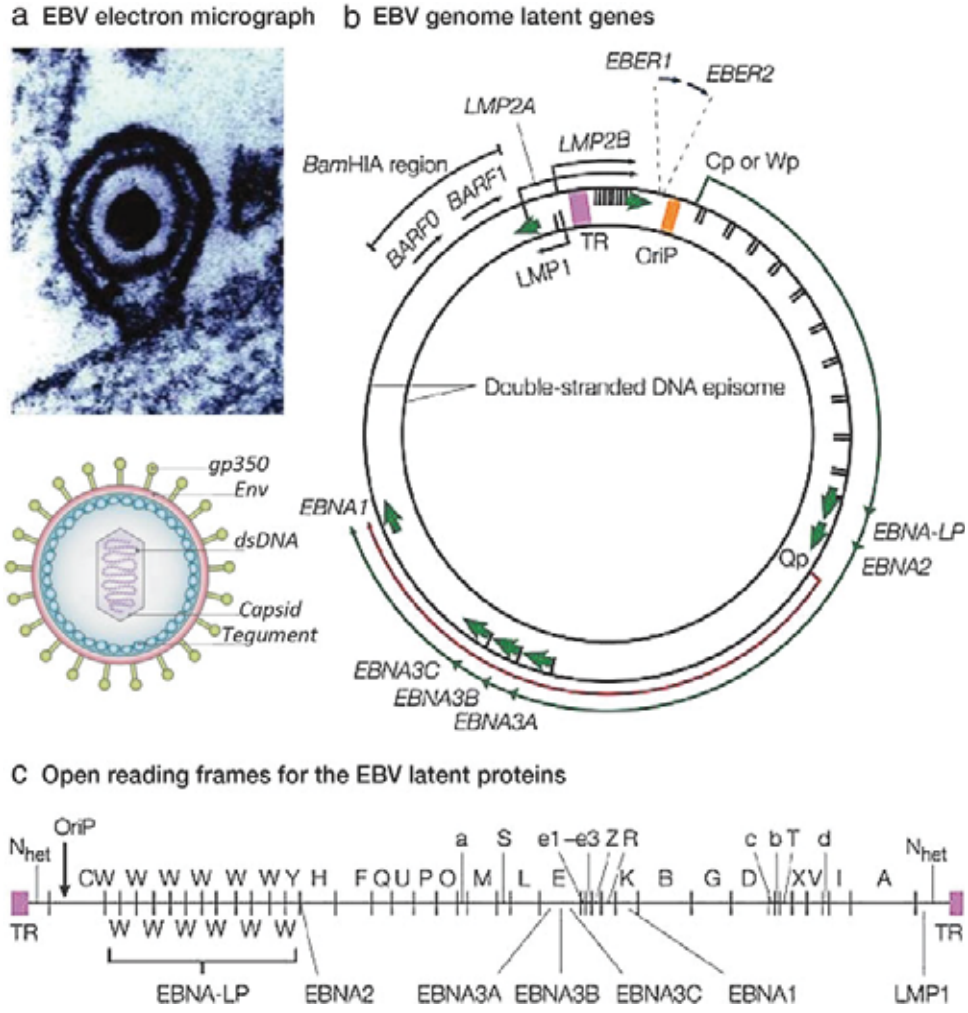

FIGURE 1 Virion composition and genome organization. (a) Electron micrograph of Epstein-Barr virus (EBV) budding from the plasma membrane (*top*). Diagram of virion components (*bottom*). (b) Diagram of circular genome (episome) with localization of latent transcripts. (c) Diagram of linear genome displaying localization of BamHI fragments. (Modified from ref. 264 with permission; virion image from Wikipedia.)

cell contains 1 to 200 nuclear copies of the circular EBV genome (known as an episome) that is attached to, but not integrated into, host chromosomes by the viral protein EBNA-1 (25). Episome amplification occurs early after infection. The terminal DNA repeat elements serve as landmarks on the EBV episome that distinguish between EBV isolates as individual viruses and tend to maintain a constant number of terminal repeats upon serial passage of latently infected cells. This finding has proved extremely useful in determining whether latently infected tumor cells, such as BL, arise from a single progenitor (26).

Antigenic cross-reactivity between EBV and other herpesviruses is rare, even among proteins encoded by conserved genes. In fact, the EBV genes expressed in latent infection as well as several lytic-cycle genes, such as the tegument genes, have no detectable homology to other herpesvirus genes and are believed to derive in part from cellular DNA (12) or possibly DNA of coinfecting pathogens. As an example, an irregular repeat motif, GGGGCAGGA, present in the latent-cycle gene EBNA-1, is also interspersed in human DNA. Some other examples of EBV lytic-cycle genes with significant homology to the human genome, but little homology to other herpesviruses, include BZLF1, BHRF1, and BARF1. BZLF1 is an immediate early gene closely related to the *fos* and *jun* transcriptional activators (27). BHRF1 is an EBV early gene with significant homology to the human *bcl-2* gene (28) involved in prevention of apoptosis. BARF1 is an early gene with amino acid sequences similar to that of c-fms (colony-stimulating factor) or the colony-stimulating factor-1 receptor (CSF-1R) (29). Many noncoding RNAs (ncRNAs) with diverse functions are produced by EBV, including EBV-encoded RNAs (EBERs), BamHI-A rightward transcripts (BARTs), a small nucleolar RNA (snoRNA), and multiple microRNAs (miRNAs) generated in particular from the BamA and BamH DNA fragments (30–32).

Host Range and Virus Receptor

EBV is restricted to humans, although related lymphocryptoviruses have been detected in both Old and New World nonhuman primate (NHP) species (33, 34). Lytic cycle proteins are the most conserved and latent cycle proteins are the most diverged between humans and NHPs. The B cells of certain NHPs, including squirrel/owl monkeys and cotton-top tamarins are susceptible to EBV immortalization *in vitro*. These animals may undergo EBV infection upon percutaneous inoculation (35, 36), but do not provide physiologic disease models; cross-species infection is essentially absent (37). Upon oral infection with rhesus lymphocryptovirus (rhLCV), but not with EBV, rhesus macaques develop a spectrum of EBV-like illnesses (34). More recently, immunodeficient mice transplanted with human immune systems, "humanized mice," were shown to be susceptible to infection and to recapitulate many forms of EBV-associated disease, providing many new avenues for the study of EBV pathogenesis (38–40).

B lymphocytes are the primary cellular reservoir of EBV infection. The initial stage of infection involves a high-affinity interaction between the major EBV outer envelope glycoprotein gp350 with CD21 on the surface of B cells (41–43). Multiple lines of evidence confirm CD21, the receptor for the C3d component of complement, as the primary human cellular receptor on B cells: (1) purified CD21 binds to EBV, (2) virus infection of B-cell lines is blocked by antibody directed against the CD21 glycoprotein, and (3) the expression of CD21 on heterologous cells confers binding to EBV. Comparison of the primary amino acid sequences of gp350 and C3d reveal a shared nonapeptide (EDPGFFNVE) that may account for some conserved receptor contacts (44). In addition to infecting B cells, EBV can variably infect other hematopoietic lineage cells, including T cells, natural killer (NK) cells, monocytes, smooth muscle cells, and others (45). CD21 can be detected on some thymocytes and peripheral blood T cells (46). CD35, a human complement regulatory protein closely related to CD21, can also bind gp350 and initiate entry (47). The cellular distribution of CD35 is broader; thus, CD35 may facilitate EBV infection of some non-B-cell targets that sometimes become infected. As discussed below, the receptor(s) used for attachment of EBV to primary epithelial cells is distinct and interactions are more complex, although CD21 transcripts are present in primary tonsil and adenoid epithelial cells, and epithelial cell lines that express CD21 protein can utilize the CD21 receptor to support latent infection efficiently (48, 49).

EBV Adsorption, Penetration, and Uncoating

gp350-CD21 attachment on B cells initiates intracellular signaling (50–52) and transfers virus toward the cell surface for envelope fusion with the plasma membrane or endocytic vesicle membranes, depending on cell origin (53). EBV envelope fusion with B-lymphocyte cell membranes is initiated by interactions between human leukocyte antigen class II (HLAII) on the B-cell surface and the virion membrane glycoprotein gp42 in complex with glycoproteins gH and gL (gH/gL heterodimer) (54). EBV glycoprotein B, gB (gp110), a highly conserved herpesvirus family glycoprotein, then functions as the viral fusogen (55).

EBV also infects epithelial cells *in vivo*, and oral epithelial cells produce high-titer transmissible virus. Studies of epithelial infection *in vitro* have proved more challenging than those of B cells due in part to the complexity of polarized epithelial structures. However, recent models using organotypic (raft) cultures, similar to those used for human papillomavirus (HPV) studies, are beginning to yield new information (56, 57). Interaction between B cells and epithelial cells may be required to promote epithelial cell infection constitutively (58), as agammaglobulinemic patients, who harbor immature B cells, lack all evidence of EBV infection (59). Moreover, EBV latency is sometimes (although rarely) eradicated following aggressive bone marrow transplantation (60). CD21-independent epithelial attachment is more effective in the relative absence of gp350 (61), which is less abundant on virions produced by B cells than by epithelial cells (62).

Whether a single predominant ligand is used to initiate attachment to all primary epithelial cells is unclear. Several attachment proteins have been proposed, including the BMRF2 protein (contains an RGD sequence that can bind β1 integrins), gH (has a KGD sequence that binds αvβ5, αvβ6, or αvβ8 integrins), and possibly gp150 (63, 64). Fusion of the EBV envelope with epithelial cell membranes occurs at the plasma membrane, does not require BMRF2, and is initiated by cell membrane interaction with gH/gL and gB in the absence of gp42. Despite the observation that fusion with both B cells and epithelial cells requires gH/gL and gB, differences in the respective fusion pathways have been described (63).

The mechanisms involved in transport of the nucleocapsid to the nuclear membrane and delivery of viral DNA are not well understood. Differences between B cells and epithelial cells have been observed *in vitro* (65). Upon dissolution of the capsid, tegument proteins and RNAs are

released into the cytosol; some of these prevent lytic cycle activation, for example, by blocking access of critical lytic cycle transactivators to the nucleus, thereby favoring viral persistence (66). Once inside the nucleus, the linear EBV genome circularizes, which precedes or at least coincides with *de novo* gene expression directed from the first latent infection promoter Wp. The EBV genome is replicated by cellular DNA polymerases during S phase of the cell cycle and persists as multiple, extrachromosomal double-stranded EBV episomes, which are organized into nucleosomes similar to chromosomal DNA (12). Epigenetic modifications occur rapidly and are critical for the establishment of infection. They are complex, varying by cell type and pattern of infection, as reviewed recently (67–69).

Latent Infection

The hallmark of B-lymphocyte EBV infection is the establishment of latency, which is characterized by three distinct processes: (1) viral persistence; (2) restricted virus expression, which alters cell growth and proliferation; and (3) retained potential for reactivation to lytic replication. Genome persistence is achieved through maintenance of multiple copies of covalently closed episomal DNA in the nucleus. The episomes are replicated semiconservatively during S phase by cellular DNA polymerases, and equal partitioning to daughter cells is mediated by interactions between the latent origin of plasmid replication (OriP) and EBNA-1 (70). The ~172-kbp EBV genome encodes between 80 and 100 genes and 30–40 ncRNAs (12, 31). Limited gene products expressed differentially during distinct latency programs are involved in establishing and maintaining the "immortalized" state. These include six EBV nuclear proteins (EBNA-1, -2, -3A, -3B, -3C, and -LP); two latent membrane proteins (LMP-1 and LMP-2A/B); two small, nonpolyadenylated RNAs (EBER-1 and -2); and multiple other forms of ncRNAs (12). *In vitro* latency can be disrupted, although inefficiently, through a variety of cellular activators that stimulate expression of BZLF1 with associated destruction of the host cell (69, 71–73). Specific mutations in BZLF1 inhibit its ability to induce lytic replication, but addition of a second protein, BRLF1, partially restores this activity.

Although only a minority of EBV genes are expressed in latently infected B cells, the transcribed regions encompass a major portion of the viral genome. Following circularization of linear EBV DNA in the nucleus, the EBNA mRNAs are assembled by alternative splicing and 3' processing of a common precursor encoded by more than 100 kb of the genome. Transcription of the EBNA and LMP genes is mediated by cell-derived RNA polymerase II, whereas EBERs are transcribed by RNA polymerase III (74). EBERs are the most abundantly transcribed EBV products in all latently infected cells (10^4 to 10^5 copies/cell), distantly followed by the LMP-1 gene, which, in turn, is significantly more abundant than the EBNAs and LMP-2 genes in LCLs (12). Figure 1b depicts the extensive transcription and long-range splicing of the EBV genome, which occurs in latently infected B-LCLs. Selection of alternate promoters, complex splicing, and enhancer looping ultimately determine the type and levels of latent gene expression in distinct cell types (75). Transcriptional regulation during latent infection has been reviewed comprehensively (76).

EBNA-2 and its coactivator EBNA-LP are the first EBV proteins expressed during latent infection of primary B cells and reach their steady-state levels within 24–32 hours. EBNA-2 is required for initial expression of other EBV latent genes and for transactivation of cellular genes in primary cells. Thus, EBNA-2 is essential for immortalization, and viruses with EBNA-2 deletions are incompetent to transform primary human B lymphocytes. By 32–96 hours postinfection, all of the EBNA proteins and LMP-1 can be detected. Concurrent with LMP-1 expression are an increase in surface CD23 and onset of cell DNA synthesis. Cellular DNA repair processes are activated upon primary B-cell infection, and typically a cell fate decision is made that determines whether immortalization will be maintained (77).

Transformation and Latent Products

Herpesvirus persistence is characterized by the ability of each family member to switch between a lytic and latent life cycle driven by individual transcription programs. Several well-defined transcription programs, known as latency 0, I, II, and III (and abortive replication), characterize the different forms of EBV latency. The latency I, II, and III programs reflect transitions that occur during the course of normal B-cell differentiation (Fig. 2) and are characteristic of different EBV-bearing tumors (Fig. 3). Many of the proteins and RNAs that participate in latency are multifunctional. Classic roles are summarized briefly below, but for each latent product further review is recommended (78).

EBNA-1

EBNA-1 is required for replication of episomes and maintenance of the viral genome. It is consistently expressed in EBV-bearing tumors as well as LCLs, where it is able to associate with all chromosomes in the nuclei of latently infected cells. The combined interaction of EBNA-1 with both OriP and chromosomal proteins ensures equal partitioning of EBV episomes to progeny cells. EBNA-1 activates other EBV latency genes and also interacts with certain cellular proteins to enhance cell survival (70, 79, 80).

EBNA-2

EBNA-2 is essential for primary immortalization of B lymphocytes and for initial expression of EBNA-1, EBNA-3, and the LMPs. EBNA-2 specifically transactivates expression of the B-lymphocyte activation marker CD23, which is abundantly expressed on LCLs and antigen-primed B lymphocytes (12). Together with its coactivator EBNA-LP, EBNA-2 is now known to transactivate cooperatively many viral and cellular genes that participate in *in vitro* immortalization of primary B cells, supporting the latency III program. EBNA-2 does not directly bind DNA, but associates with other proteins that regulate transcription (81), such as cellular CBF1, to bind to both promoter and enhancer regions of predominantly B-cell regulatory factors.

EBNA-LP

EBNA-LP (EBNA-5), or leader protein, and EBNA-2 are the first latent proteins expressed upon primary B-cell infection. Transcription of EBNA-LP is complex and multiple isoforms are typically produced. EBNA-LP primarily functions as an EBNA-2 coactivator. However, like EBNA-2, EBNA-LP cannot bind DNA directly. When expressed independently, EBNA-LP activation is limited to promoter regions. Although the functions of EBNA-LP remain unclear, roles in modulating cellular repressors and in prevention of apoptosis early after infection are under investigation (81).

EBNA-3

EBNA-3 consists of a family of three high-molecular-weight gene products—EBNA-3A, -3B, and -3C (also known as

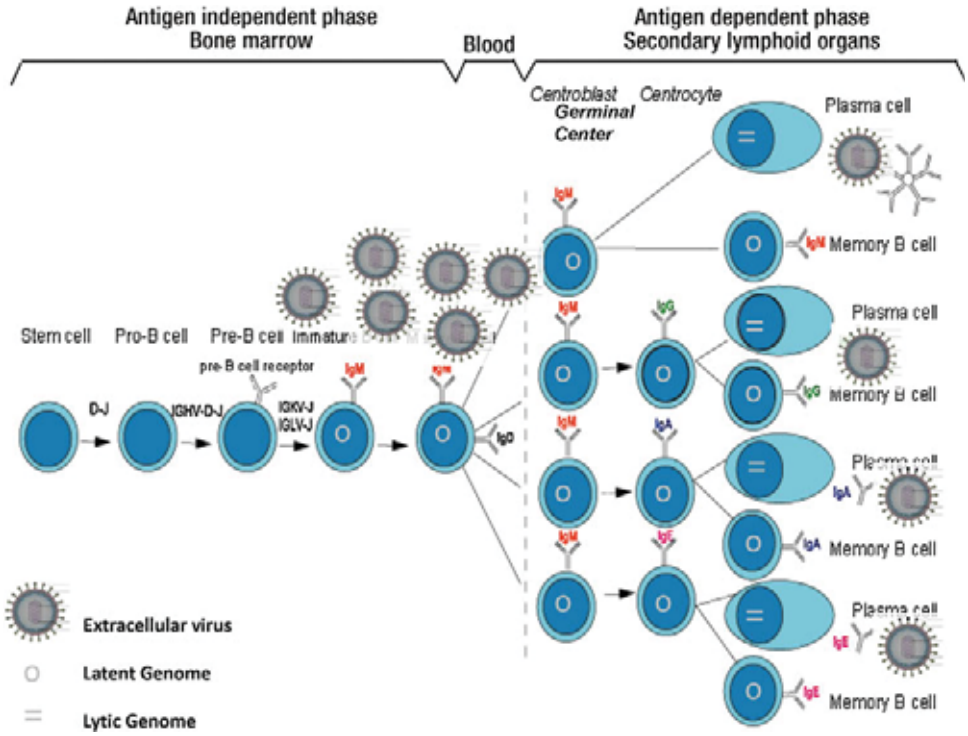

FIGURE 2 Following primary infection, Epstein-Barr virus (EBV) in a latent state accompanies its B cell host through the B lymphocyte maturation process, only switching to the lytic cycle and virus production upon plasma cell (terminal) differentiation. (Diagram of B cell differentiation from Quizlet.com and modified by the author to reflect virus biology.)

EBNA-3, -4, and -6)—that are located in tandem on the EBV genome (see Fig. 1). Like the EBNA-2 gene, the EBNA-3 genes are polymorphic transcription factors that vary in EBV-1 and EBV-2, although type specificity in contrast to EBNA-2 does not appear to affect the ability of the virus to initiate growth transformation *in vitro* (17). Analysis of the immortalization capacity of EBV recombinants with specific mutations in each of the EBNA-3 genes has demonstrated that while EBNA-3B is dispensable for B-lymphocyte transformation *in vitro*, deletion of either EBNA-3A or -3C renders EBV transformation incompetent (82). Whereas EBNAs 3A and 3C operate as oncoproteins, EBNA-3B, in contrast, may function as a tumor suppressor, a role best demonstrated upon analysis of tumor models *in vivo* (83).

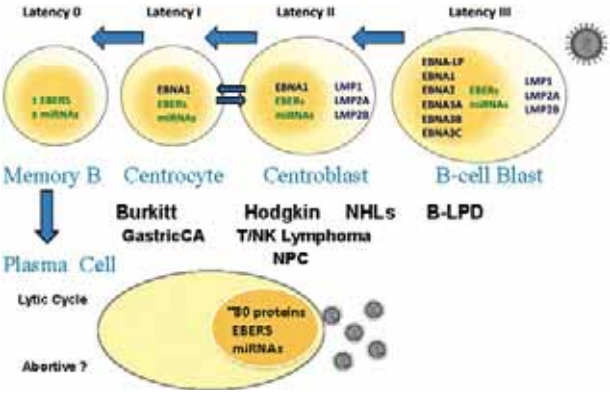

FIGURE 3 Distinct Epstein-Barr virus (EBV) expression programs (latency III, II, I, 0, lytic replication, abortive replication) are linked with defined stages of B lymphocyte maturation. These same latency patterns are detected in the B cell tumors that arise from transformed cells blocked from further differentiation. Major classes of B cell cancers are indicated in bold. Other EBV-associated tumors that share the indicated latency program but are not of B cell origin are shown in plain font.

LMP-1

LMP-1, the second most abundant EBV mRNA in latently infected B lymphocytes (~60 copies/cell), encodes a 63-kDa, multiple-pass, integral membrane protein. A truncated form of LMP-1 is expressed during lytic replication. Full-length LMP-1 functions as a constitutively active receptor and is the major transforming gene product of EBV. In early studies, transfer of LMP-1 into continuous rodent fibroblast lines was found to elicit multiple transformation-associated changes and to promote development of xenograft tumors in nude mice (12, 84).

Although EBNA-2 upregulates LMP-1 in B cells, LMP-1 is expressed in the absence of EBNA-2 during B-cell differentiation and in epithelial as well as several lymphoid tumor types. LMP-1 is essential for B-cell immortalization (LCL formation) *in vitro* (12). Transfer of LMP-1 into EBV-negative cells induces surface expression of a number of molecules involved in activation of B cells (e.g., CD23, CD39, CD40, CD44) and epithelial cells (e.g., EGFR). LMP-1 also protects EBV-infected cells from programmed cell death (apoptosis) through signaling that results in induction of the cellular oncogene *bcl-2* and blocks terminal differentiation. Mice expressing an LMP-1 transgene driven by an immunoglobulin promoter develop B-cell lymphomas, and keratin 14–driven LMP-1 is a modest tumor promoter in

transgenic mice. LMP-1-bearing B cells grow in tight clumps due to increased expression of homotypic cellular adhesion molecules, and LMP-1 induction of the adhesion molecules lymphocyte function–associated antigen 1 (LFA-1), LFA-3, and intercellular adhesion molecule 1 (ICAM-1) promotes interaction between B and T lymphocytes via the LFA-3/CD-2 and LFA-1/ICAM-1 adhesion pathways, as well as others. These heterotypic adhesions are important because *in vivo* elimination of EBV-transformed B lymphocytes is dependent on conjugate formation with cytotoxic T cells. The signaling response induced by LMP-1 partially mimics that induced by CD40 ligation (85) and occurs through engagement of tumor necrosis factor receptor (TNFR) family ligands with the LMP-1 carboxyl terminus. The diverse functions of LMP-1 involve multiple signaling pathways [nuclear factor-κB (NF-κB), mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3-K)/AKT, interferon regulatory factor 7 (IRF7), and Janus kinase/signal transducers and activators of transcription (JAK/STAT pathways] (86). Mutations, particularly in the intracellular carboxyl terminus of LMP-1, can eliminate signaling and prevent transformation (87).

LMP-2

LMP-2 is an integral membrane protein containing 12 hydrophobic transmembrane domains that localize to cell membranes and can colocalize with LMP-1 in the plasma membrane. Two major isoforms LMP-2A and an amino-terminal truncated isoform LMP-2B predominate. Among the transformation-associated EBV proteins, LMP-2 is not required for establishment of LCLs *in vitro*, but appears to be critical for EBV persistence *in vivo* because it is retained in several EBV-bearing tumors (Fig. 3). The cytoplasmic amino terminus of LMP-2A contains multiple motifs, including several phosphorylated tyrosines, an immunoreceptor tyrosine-based activation motif (ITAM), and proline-rich motifs that interact with and modulate multiple host cell proteins involved in signaling. LMP-2A, but not LMP-2B, subverts normal signal transmission through the B-cell receptor, blocking terminal differentiation by substitution with tonic activation signals that promote B-cell survival and prevent apoptosis. Transgenic mice expressing the LMP-2 transgene under immunoglobulin regulation exhibit increased survival of normal B cells (88). LMP2A expression in epithelial cell lines increases spreading, migration, and clonability in soft agar. In raft cultures, blockade of epithelial cell differentiation and hyperproliferation is observed. LMP-2A and -2B together with EBNA-1, LMP-1, and miRNAs (latency II) are most consistently detected in nasopharyngeal carcinoma (NPC) and EBV-bearing (HLs)'s lymphomas, but can be expressed in other EBV-associated malignancies as well (89, 90).

EBV-Encoded RNAs—EBER-1 and EBER-2

The most abundant EBV transcripts in latently infected B cells are the small (167- and 172-nucleotide), noncoding, nonpolyadenylated RNAs termed EBER-1 and EBER-2. Unlike other EBV genes expressed during latent infection, the EBERs are also transcribed during lytic infection. EBV-infected cells typically express 1–5 x 10^6 EBER copies/cell, and detection of EBERs by *in situ* hybridization is a widely used technique to detect EBV-infected cells in clinical samples. The majority of EBERs are localized within the cell nucleus, where they are complexed with the cellular protein La. Additional interactions with cellular proteins including Pax5 (EBER-2) and L22, heterogeneous nuclear ribonucleoproteins (hnRNPs) (EBER-1) have been reported. Despite their prevalence, the role of EBERs in infection remains largely unknown, although different lines of evidence suggest functions related to type I interferon (IFN) regulation. The EBERs are not required for immortalization of primary B cells *in vitro* (91); however, the observation that EBER sequences are highly conserved across EBV strains suggests they are vital to virus persistence *in vivo* (92, 93).

ncRNAs and miRNAs

The precise role of the recognized 30–40 EBV ncRNAs, in addition to the EBERs, remains largely unknown. EBV encodes a single snoRNA of ~65 nucleotides localized to the BART region. The V-snoRNA1 binds RNPs and is proposed to guide RNA modification. A stable abundantly expressed (comparable to EBER-2) 81-nucleotide EBV–small interfering RNA (siRNA)-1 derived from an intron within the W repeats has been identified recently in latently infected cells, as has a second novel RNA able to form a massive 586-nucleotide hairpin. No functional information is currently available. A family of alternatively spliced, polyadenlyated and processed RNAs that arise from the BamHI-A region (BARTs) predominantly localize to the nucleus. They are abundant in EBV-bearing epithelial tumors and less so in EBV-bearing lymphoid neoplasms. They may function similarly to cellular long ncRNAs (lncRNAs) in regulation of viral or cellular gene expression.

The very existence of viral miRNAs was first demonstrated in EBV in 2004. miRNAs are small (~22 nucleotides) ncRNAs that regulate gene expression posttranscriptionally, each altering as many as 200 transcripts. Approximately 30–40 miRNAs have been identified primarily in latently infected cells, but some in lytically infected cells, and appear to variably alter both viral and cellular transcripts. Most are encoded by introns in the BART region and a smaller number adjacent to BHRF1 sequences (Fig. 4). Diverse studies from this rapidly evolving area indicate that several EBV miRNAs contribute to EBV-driven B-cell immortalization *in vitro* as well as to the development of specific cancers (31, 94, 95).

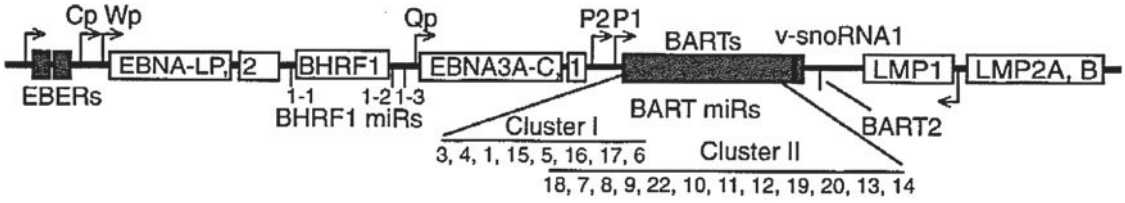

FIGURE 4 Genomic origin of noncoding RNAs including EBV-encoded RNAs (EBERs), microRNAs (miRNAs), BamHI-A rightward (BART), and an Epstein-Barr virus (EBV) small nucleolar RNA (snoRNA). There is clustering of precursor miRNAs in the BART and to a lesser degree in the BHRF1 region of the genome. (Reproduced from ref. 31 with permission from the journal.)

EBV DNA Persistence in Latency

During primary infection, EBV sequentially employs distinct transcription programs (see Fig. 3), initially triggering the formation of B-cell blasts that then transit to germinal centers where the EBV-infected B cells undergo switch recombination and somatic hypermutation and emerge as resting latently infected memory B cells (see Fig. 2), the primary reservoir for lifelong EBV infection (96). In all, 25% to 50% of peripheral blood memory B cells are infected latently during primary infection and manifested as AIM. The frequency of latently infected primary B cells falls to 1 in 10^5 to 1 in 10^6 during persistent infection (97). Latently infected memory B cells typically contain 1 to 20 EBV episomes per cell. These memory B cells are transcriptionally quiescent and express only small amounts of EBNA-1, required for episomal maintenance upon cell division; thus, they remain undetectable during immune surveillance. Periodic activation and differentiation of memory B cells into plasma cells initiates the EBV lytic replication cycle (98). EBV-specific $CD8^+$ and $CD4^+$ T cells are important in eradicating these cells and controlling new cycles of infection (99).

Lytic Infection and Virus Replication

Lytic transcripts are required not only for transmission through production of virions released into saliva, but also directly contribute to primary infection of B cells. *In vitro*, latently infected B cells can be induced to undergo lytic-cycle replication by activation with biological and chemical inducers, including phorbol esters, stress inducers, calcium ionophores, and various genome-modifying agents, or by cross-linking cell surface immunoglobulin (Ig). Cross-linking of membrane Ig by cognate antigen on the surface of memory B cells *in vivo* is believed to constitute the major physiologic signal that, upon stimulation of plasma cell differentiation, initiates epigenetic modifications of the EBV genome required for lytic cycle induction (100). Following induction, host cell protein synthesis is shut off. Induced cells undergo cytopathic changes characteristic of lytic herpesvirus infection, including chromatin margination, viral DNA synthesis, nucleocapsid assembly at the nucleus periphery, and virus budding through the nuclear membrane (12).

During lytic EBV infection, immediate early genes are defined as genes that are transcribed in newly infected cells in the absence of *de novo* viral protein synthesis. The key immediate early transactivators of EBV lytic-cycle genes are the 1-kb BZLF1 (Zta) messenger RNA (mRNA) and the 2.8-kb BRLF1 (Rta) RNA. Early (E) genes ($\sim$30 proteins), such as the thymidine kinase and polymerase complex, function in viral DNA replication. The induction of EBV DNA synthesis is preceded by an increased episome copy number that is consistent with a rolling circle replication model. Long concatemers of linear EBV genomes are synthesized, cleaved, and then packaged into viral capsids. The EBV genes expressed during the late (L) stages of lytic infection ($\sim$30) primarily encode tegument and structural proteins that mediate virion formation and egress (101). Genes that encode several immunoevasins are synthesized in the course of lytic replication (102, 103).

EPIDEMIOLOGY

Distribution and Geography

Seroepidemiological studies show that antibodies to EBV are present in all population groups and about 90% to 95% of adults worldwide are EBV seropositive. In lower-resource settings, most children acquire EBV infection during the first 2 years of life; for example, 82% of children in Ghana were EBV seropositive by 18 months of age (104, 105). In higher-resource settings, EBV infection typically occurs in late childhood, adolescence, or early adulthood (106, 107). With global development, EBV acquisition has become increasingly delayed worldwide (106, 108).

Incidence and Prevalence of Infection

The incidence of clinically symptomatic infection AIM is greatest when primary EBV infection is delayed until the second decade of life. In the United States and Great Britain, EBV seroconversion occurs before age 5 in about 50% (20% to 80%) of the population, depending on age, location, race/ethnicity, and socioeconomic factors. The overall incidence of AIM in the United States is about 50 cases/100,000 per year, with the highest incidence in the 15- to 24-year age group. In college-aged populations, 30% to 75% of entering students are EBV seronegative (109). Most susceptible individuals become infected during their freshman year, and >75% of primary infections may be associated with AIM (110, 111). By contrast, AIM is observed in fewer than 10% of primary infections among infants and children, and this discrepancy is currently a major focus of investigation. No obvious yearly cycles, seasonal changes, or sex differences have been noted in relation to AIM incidence (106). AIM is a risk factor for subsequent development of EBV-positive HL, which, because it is associated with AIM, is also increasing among young adults coordinately with global development (112).

EBV is most consistently associated with two endemic malignancies, childhood BL (equatorial Africa) and NPC (southeast Asia) in addition to sporadic B-lymphoid cancers typically linked to cellular immunocompromise (12). The epidemiologic factors that contribute to individual malignancies are discussed below.

Transmission

EBV is transmitted primarily through contact with infectious oropharyngeal secretions. Virus in saliva is the major route of transmission from mother to child, although breast milk also contains infectious virus (113). Siblings and playmates are additional sources of infectious oropharyngeal secretions (114, 115). Transmission among adolescents and young adults is attributed to deep kissing (107, 110). Whereas detection of EBV in the uterine cervix suggests that sexual transmission can sometimes occur (116), peripartum transmission is extremely rare and description is limited, likely because of the high prevalence of protective antibody among reproductive-age women (117). Acquisition through blood transfusion and following solid organ transplant (SOT) and hematopoietic stem cell transplant (HSCT) has also been documented.

High viral titers are present in throat washings of 100% of patients during primary infection (99, 118), whereas intermittent, asymptomatic oropharyngeal shedding persists at lower levels for the lifetime of infected individuals (12% to 25%). Immunocompromised patients typically shed the virus between 50% and 90% of the time (119). As a consequence, only a small percentage of patients with AIM recall prior contact with other individuals with AIM, and most infections are acquired from asymptomatic shedders.

PATHOGENESIS

Initiation of Infection

B lymphocytes that underlie the tonsillar epithelial crypts are thought to be the initial cells infected *in vivo* (120, 121),

because persons who lack B cells or whose B cells are immature are not susceptible to infection, even of the epithelial cell compartment (122). Productive infection of oropharyngeal B cells followed by epithelial cell transmission leads to amplification of lytic virus replication, release of EBV into salivary secretions, and infection of additional B cells in the lymphoid-rich areas of Waldeyer's ring. As noted, memory B cells are likely responsible for dissemination of the infection throughout the lymphoreticular system and are necessary for EBV persistence (Fig. 5).

Incubation Period and Early Infection

The incubation period from initial exposure to symptoms is approximately 6 weeks and coincides with the onset of high viral loads detected by PCR in both saliva and blood (111, 123). Large quantities of infected B cells (up to 25% to 50% of memory B cells) can be detected in the circulation upon onset of symptoms (106). The cellular immune response to infection is massive, with extraordinary expansion of CD8 T cells, inversion of the CD4/CD8 ratio, and cytokine storm (124). It is likely that this response not only limits further rounds of viral replication and EBV-positive B-cell expansion, but also directly contributes to many symptoms of AIM associated with the abundant release of inflammatory cytokines (Fig. 5). Perturbations of early cellular immune responses, especially in persons with congenital or acquired immunodeficiency, can result in atypical or uncontrolled EBV infection, including rapid development of EBV-induced malignancy, as described in later sections.

Humoral Immune Responses

Primary EBV infection induces circulating antibodies directed against viral antigens as well as unrelated antigens found on sheep (heterophile) and horse (monospot) red blood cells. These heterophilic antibodies represent a heterogeneous group of mostly IgM antibodies that do not cross-react with EBV antigens. The detection of heterophile antibodies was used for many years to screen patients with symptoms of AIM for new infection, but has been largely discontinued due to lack of sensitivity (low in young children) and specificity (can be positive in liver disease, lymphoma, autoimmune disease, others) (125). Current diagnosis of acute EBV infection is most often based on indirect antibody immunofluorescence testing (IFT) or modifications thereof. Serum IgM against the viral capsid antigen (VCA) complex is typically detected 2 days after the onset of clinical illness and is followed by stable conversion to IgG anti-VCA. Although the IgM response to VCA reaches high titers early, it disappears within several weeks. IgM VCA antibodies are not detected in the general population, and thus their presence is virtually diagnostic of primary EBV infection. IgG antibody titers to VCA reach peak levels 2–4 weeks later and persist thereafter at lower levels for life. IgG directed against early lytic-cycle replication-related complexes (early antigen, EA) tends to appear with the peak IgM VCA response but reaches maximal levels after the IgM response. IgM antibody to EA may actually appear before antibody to VCA, but is not routinely used in clinical diagnosis. IgG anti-EBNA-1 titers do not usually

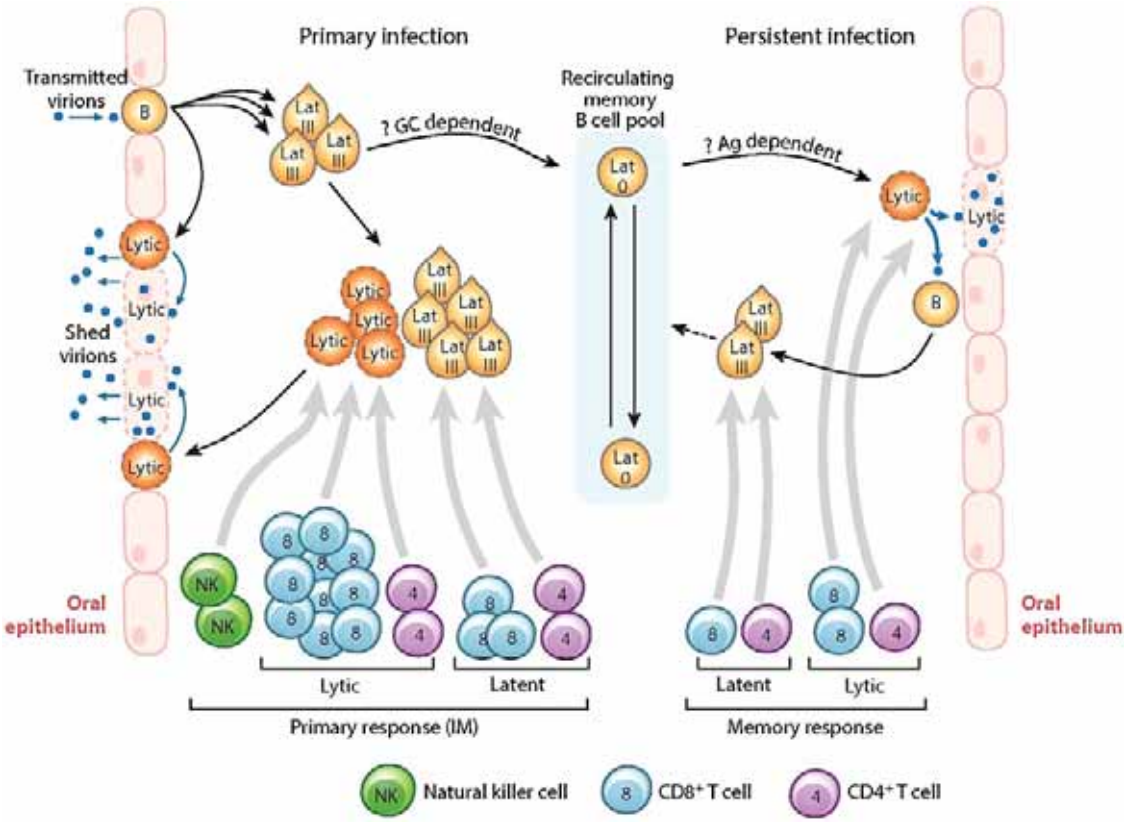

FIGURE 5 Immunobiology of Epstein-Barr virus (EBV) infection in the normal host. Diagram illustrating the transmission, primary infection, persistent infection, and how the cellular immune response becomes activated to prevent disease. (Reproduced from ref. 151 with permission from the journal.)

develop until convalescence, and not all persons develop these antibodies (126).

Immunoblot profiling of EBV antibodies is now used for a number of applications and has recently reached commercial platforms (127). Normal EBV carriers develop and maintain a highly similar anti-EBV IgG antibody titer including antibody diversity patterns, and deviations from these patterns can predict reactivation of infection (e.g., increases in EA) as well as development of disease (rising and more diverse IgG VCA and EA antibodies in chronic active EBV) and NPC (rising and increasingly diverse IgG and IgA VCA and EA) (128). Dramatic increases in antibody titers often occur in the setting of T-cell compromise, suggesting that this is a compensatory mechanism, although how it is triggered is unknown.

In addition to virus-specific antibodies assessed for diagnostic purposes, IgG responses are frequently detected to EBV early lytic antigens (BRRF1 and BORF2) and to tegument proteins (BBRF, BGLF2, BNRF1) (129). Although gp350 is the most abundant viral protein in lytically infected cell plasma membranes, and most of the human EBV-neutralizing IgG antibody response is directed against gp350 (130–132), neutralizing antibody development is typically delayed, reaching detectable levels only 6–7 weeks after the onset of illness and peak levels many weeks later (133, 134). Stable anti-gp350 titers then persist for life. Gp350-specific antibodies can mediate complement fixation and antibody-dependent cellular cytotoxicity (135). Because the gp350 molecule is conserved between EBV-1 and -2 strains, gp350 is considered to be an essential component of a prophylactic EBV vaccine. Figure 6 depicts the onset and time course of peak EBV-specific antibody responses observed in young adults with AIM in clinical practice.

Individual humoral response to EBV proteins, particularly to EBNA-1, may result in cross-reaction with cellular components. In persons who are genetically predisposed, these cross-reactive antibodies have been implicated by some in the pathogenesis of autoimmune diseases including multiple sclerosis (MS), systemic lupus erythematosus (SLE), rheumatoid arthritis, and Sjögren's syndrome, but without rigorous confirmation (136, 137).

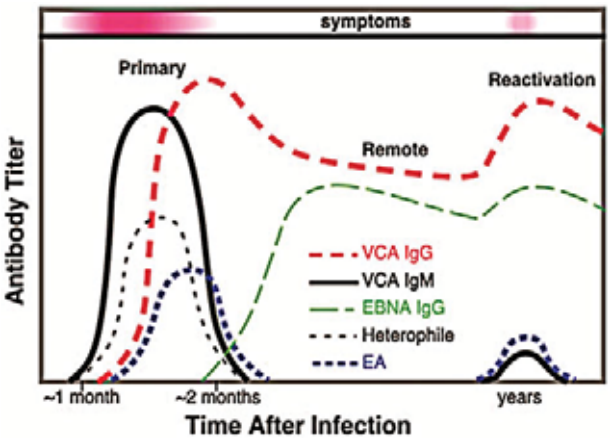

FIGURE 6 Classic Epstein-Barr virus (EBV) serologic responses in acute versus remote versus reactivated EBV infection. (Reproduced from ref. 238 with permission from the journal.)

Cellular Immune Responses

NK cells

Because primary pediatric infection is often asymptomatic or undiagnosed, understanding the cellular immune response that follows EBV transmission is largely based on evaluation of patients with AIM. Innate NK cells play a primary role in immune surveillance against virus-infected or transformed cells, because they do not require priming or prior antigen exposure. NK cells alone are not sufficient to prevent the establishment of EBV-transformed B-cell lines *in vitro*, but they do contribute to improved LCL regression in the presence of EBV-specific $CD8^+$ T cells (138). This effect may be due to direct cytotoxicity or IFN production, since EBV and EBV-infected cells induce IFN-γ production by NK cells (139), and IFN-α, an NK product, is known to inhibit EBV-induced B-cell proliferation *in vitro* (140). One patient with complete absence of $CD16^+$ NK cells, however, experienced an unremarkable EBV infection following life-threatening infections with varicella-zoster virus, cytomegalovirus (CMV), and herpes simplex virus (HSV) (141), suggesting that not all NK cell subsets are equally required for resolution of primary EBV infection (142). More recently, evaluation in tonsillar tissues, modeling in humanized mice, and studies of young children have again emphasized the importance of NK cells, specifically NK subsets, in controlling EBV infection. As one example, a $CD56^+$, $CD16^-$ NK cell subset, which progressively declines as children age, is highly effective in eliminating lytically infected cells *in vitro* and therefore has been proposed to mitigate the exuberant CD8 T-cell response observed among adolescents with AIM (143, 144).

Other Innate Immune Cells

Precisely how dendritic cells, monocyte/macrophages, and granulocytes contribute to prevention of primary infection and to EBV-associated disease is an area of active investigation. EBV encodes multiple products (proteins, ncRNAs) believed to function in evasion of innate immunity, including secreted products that are incorporated as exosomes (145). Interactions with Toll-like receptors have been reported both to inhibit or promote EBV infection. In a primate model, infection with an EBV ortholog deleted in BARF1, a decoy receptor for macrophage colony-stimulating factor (M-CSF, a cytokine required for monocyte/macrophage differentiation) limited virion production, although precisely how this cell type functions in relation to EBV infection is not well understood (146). Recently, profound depletion of circulating plasmacytoid dendritic cells, and to a somewhat lesser extent conventional dendritic cells, was shown to accompany AIM, and consistent with this observation, IFN-α was minimally detected in blood (147). The mechanisms underlying this profound though transient dysregulation of the innate immune response are not presently known.

T cells

A hallmark of AIM is the appearance of "atypical lymphocytes" (primarily CD8 T cells) in peripheral blood, which account for 60% to 70% of the total white cell count that averages 12,000–18,000/mm^3. Accumulating evidence suggests that the exaggerated T-cell response seen with delayed infection may in part represent activation of cross-reactive T cells, a consequence of exposure to previously encountered pathogens (heterologous immunity) (148). Several lines of evidence indicate that EBV-specific $CD8^+$ and also $CD4^+$ T-cell responses are critical for limiting primary infection and

controlling persistent infection (see Fig. 5). EBV-specific $CD8^+$ and $CD4^+$ T cells can prevent the transformation of B lymphocytes *in vitro*. The increased incidence of EBV-associated lymphoproliferative disorders or lymphomas in individuals with compromised cell-mediated immunity [human immunodeficiency virus (HIV)-infected patients, immunosuppressed transplant recipients] also indicate that T cells are important for the long-term control of EBV replication. In fact, virtually every congenital immunodeficiency that predisposes to development of EBV-associated disease is characterized by dysregulation within the T/NK-cell limb of the immune response (149–153).

Over the past decades, powerful and precise methods for the enumeration and characterization of virus-specific $CD8^+$ and $CD4^+$ T cells in the peripheral blood have been developed (154, 155) (see Chapter 16). These include the use of labeled major histocompatibility complex–peptide complexes (tetramers) to directly detect, measure, and isolate antigen-specific lymphocytes and assays to detect antigen-specific cytokine-secreting cells following *in vitro* stimulation (IFN-γ enzyme-linked immunospot assays and flow cytometry-based assays to measure intracellular cytokine secretion). These novel methods have markedly improved knowledge of the strength and breadth of EBV-specific $CD8^+$ and $CD4^+$ T-cell responses over the course of primary and persistent infection (156).

During primary EBV infection, high frequencies of EBV-specific $CD8^+$ and $CD4^+$ T-cell responses have been detected directly *ex vivo* (157–159). EBV-specific $CD8^+$ T-cell responses in primary infection are directed primarily against lytic-cycle antigens with greater responses to immediate early and early antigens than late antigens as viral immune evasion mechanisms become sequentially activated; latent antigen-specific responses are detected later and at lower frequencies (Fig. 7) (158). Up to 25% to 44% of peripheral blood $CD8^+$ T cells are EBV specific and express CD45RO, human leukocyte antigen–antigen D related (HLA-DR), and CD38, suggesting high-level activation and turnover of these cells *in vivo* (157). In contrast, early EBV-specific $CD4^+$ T-cell responses broadly target lytic antigens as well as latent antigens (159). In persistently infected individuals, EBV-specific $CD8^+$ T cells decrease, representing up to 5% of peripheral blood $CD8^+$ T cells, whereas EBV-specific $CD4^+$ T cells are detected at much lower frequencies (up to 0.1% to 1.5%). The numbers and/or function appear to decline with old age.

CLINICAL MANIFESTATIONS

Congenital and Perinatal EBV Infections

Intrauterine infection with EBV appears to be a very rare event. Primary infection during pregnancy is unusual because in most populations fewer than 5% of pregnant women are susceptible. Nevertheless, isolated cases of infants born with congenital anomalies (biliary atresia, congenital heart disease, hypotonia, micrognathia, cataracts, and thrombocytopenia) have been attributed to primary maternal infection (160). However, a number of lines of evidence argue against EBV as a significant cause of congenital infection. Studies of large numbers of children with congenital anomalies have failed to confirm EBV infection. Furthermore, cord blood samples from thousands of infants have yielded virtually no evidence of EBV-infected or EBV-transformed cells. Finally, prospective studies on seronegative susceptible pregnant women have generally failed to verify either maternal infection or congenital abnormalities in infants of women who did develop primary EBV infection during pregnancy (161).

Primary EBV Infection in Infants and Children

Primary EBV infections in young infants, children, and some adolescents are usually asymptomatic. When symptoms do occur, they are usually mild and nonspecific (otitis media, diarrhea, abdominal complaints, and upper respiratory infection); classical manifestations of AIM, such as lymphocytosis and fever, are rare (162). In one series, blood smears from only 32 of 200 children under 4 years of age who presented with clinical findings compatible with AIM (pharyngitis and significant cervical adenopathy) had more than 50% mononuclear cells and more than 10% atypical lymphocytes (163). Respiratory symptoms were frequently prominent, especially in young infants. IgM antibodies to VCA were detected 60% of the time in infants compared to

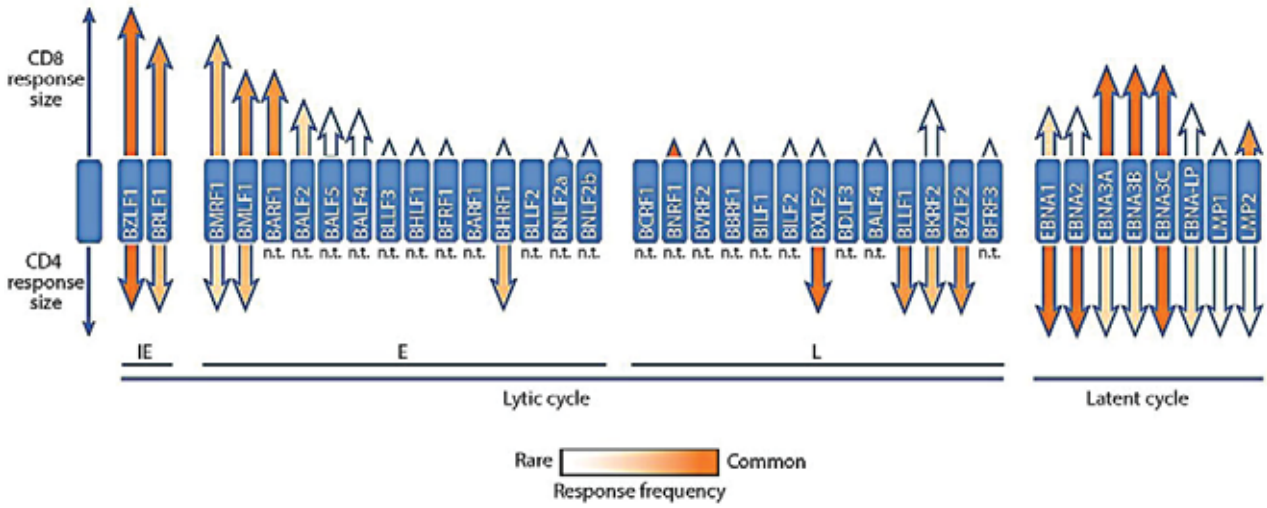

FIGURE 7 Frequency and distribution of CD4 and CD8 T cells in response to lytic and latent cycle Epstein-Barr virus (EBV) proteins. (Reproduced from ref. 151 with permission from the journal.)

100% in older children and young adults; in addition, peak titers of IgG VCA antibodies were lower, and the development of antibodies to EA was less common in infants. Prospective studies of young children demonstrate that at the time of primary infection viral loads in blood are similar to those of older individuals. Despite the reduction in antibody production, young infants mount robust EBV-specific $CD8^+$ T-cell responses during acute EBV infection, and lytic and latent proteins recognized are identical to those recognized by young adults (164). However, evidence of global T-cell activation and cytokine release, as observed in AIM, is limited.

Acute Infectious Mononucleosis

EBV-associated AIM is marked by malaise, fever, fatigue, pharyngitis, and cervical lymph node enlargement (165). Although other pathogens may produce a similar constellation of symptoms [e.g., streptococcal pharyngitis, adenoviral pharyngitis, primary CMV or human herpesvirus 6 (HHV-6) infection, acute HIV infection, toxoplasmosis, lymphoma] specific clinical as well as laboratory findings (see below) support a diagnosis of EBV-positive AIM. The lymphadenopathy associated with AIM characteristically is symmetrical and involves the posterior cervical chain more than the anterior chain; inguinal and axillary adenopathy increase the likelihood of AIM. Tonsillar exudates are a frequent component of the pharyngitis; the exudates can have a white, gray-green, or necrotic appearance (Fig. 8); palatine petechiae are often visible (166). Severe fatigue is typically prominent, and other, less common findings include rhinitis, periorbital or palpebral edema, and maculopapular and morbilliform rash. Nausea, vomiting, and anorexia often occur in patients with AIM, probably reflecting the mild hepatitis found in about 90% of infected individuals; however, hepatomegaly and jaundice are uncommon. Splenomegaly is almost universally detected by sonography, but is detected in fewer (20% to 50%) AIM patients by palpation (166).

Many patients with AIM caused by EBV have prominent pharyngeal symptoms; however, there are several other forms of the illness. Some individuals present with the so-called glandular form of the disease, in which lymph node enlargement is out of proportion to the pharyngeal symptoms. Others develop a systemic form of the infection in which fever and fatigue predominate, while lymphadenopathy and pharyngitis are mild or absent. Some patients have hepatitis in the absence of other typical features of AIM. The vast majority of individuals with AIM recover uneventfully and develop durable immunity. Acute symptoms typically resolve in 1–2 weeks, but fatigue may persist for months (106, 111).

Acute Complications

Numerous other manifestations have been associated with primary EBV infection. Neurologic syndromes can include Guillain-Barré syndrome, facial nerve palsy, meningoencephalitis, aseptic meningitis, transverse myelitis, peripheral neuritis, cerebellitis, and optic neuritis. Hematologic abnormalities, including hemolytic anemia, thrombocytopenia, neutropenia, aplastic anemia, thrombotic thrombocytopenic purpura/hemolytic-uremic syndrome, and disseminated intravascular coagulation, can occur. EBV can affect virtually any organ system and has been associated with such diverse disease manifestations as pneumonia, myocarditis, pancreatitis, mesenteric adenitis, myositis, glomerulonephritis, genital ulceration, and others comprehensively reviewed in a classic book by R.S. Chang (167).

One of the more common complications of AIM is a morbilliform rash following the administration of ampicillin or amoxicillin, and, to a lesser extent, penicillin and other antibiotics. The incidence initially was reported to be as high as 70% to 90% but is probably far lower (168). The mechanism responsible for this rash is not well understood. Development of this rash during AIM does not presage a true ampicillin allergy because patients have subsequently tolerated ampicillin without adverse reactions.

Splenic rupture is a rare but potentially life-threatening complication of AIM, estimated to occur in one or two cases per thousand (169). Almost all cases have been in males. Splenic rupture is often the first symptom of AIM that brings the patient to seek medical attention; it is spontaneous in more than half of the reported cases, with no history of specific injury. Rupture has occurred between the 4th and

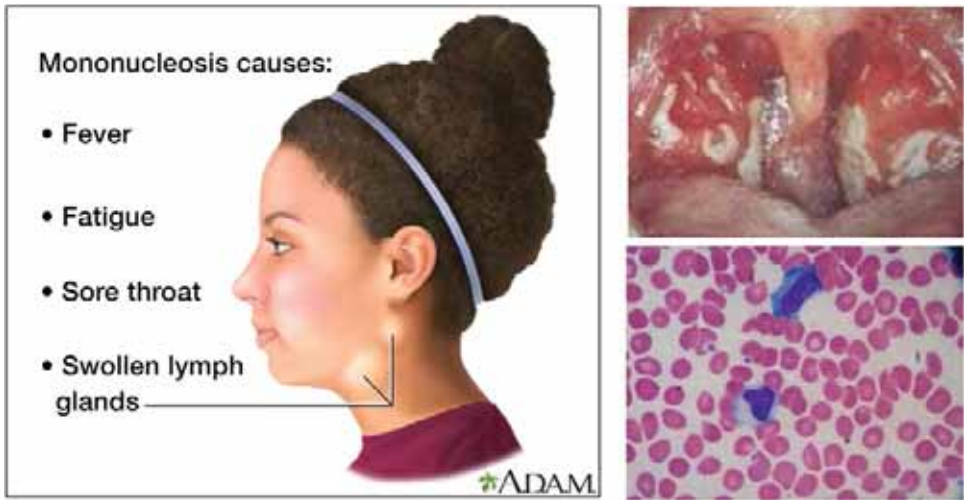

FIGURE 8 Acute infectious mononucleosis (AIM). Swollen lymph nodes, pharyngitis, fatigue, and headache comprise the four classic symptoms of AIM (*left*). Pharyngitis is often exudative (*top right*), and atypical lymphocytes are present in blood (*bottom right*). (Image of symptoms of AIM https://www.nim.nih.gov//medlineplus/ency/imagepages/17267. Image of atypical lymphocytes from Wikipedia; image of pharyngitis from ref. 265 reproduced with permission from the publisher.)

56th days of symptomatic illness and has not correlated with the clinical severity of AIM or with laboratory findings. Despite its life-threatening potential, fatality from this complication is rare. The management of splenic rupture is similar to that of other forms of splenic injury. Nonoperative treatment with intense supportive care and splenic preservation has been successfully carried out in some cases, whereas others require splenectomy (170).

Obstruction of the upper airway due to massive lymphoid hyperplasia and mucosal edema has long been recognized as an uncommon, although potentially fatal, complication of AIM. Severe obstruction can be successfully treated by tracheotomy or endotracheal intubation. Alternatively, the brief use of corticosteroids to reduce pharyngeal edema and lymphoid hypertrophy is advocated for individuals with incipient obstruction despite concerns about corticosteroid therapy in the course of severe virus infection (171).

EBV-Associated Lymphoproliferative Disorders

Atypical manifestations of EBV infection are often observed in persons with primary (congenital) immunodeficiency disease, particularly those involving the T and NK cell limbs of the immune response. The list of genetic lesions has increased greatly with advanced sequence analysis. Symptoms may be limited to EBV infections or reveal susceptibility to multiple infectious diseases (151, 153, 172). Knowledge of the disease mechanisms involved has expanded the knowledge of how lifelong asymptomatic carriage of EBV is maintained. Atypical manifestations of EBV infection may also represent a serendipitous confluence of environmental factors, for example, transient coinfection facilitating EBV entry into otherwise nonpermissive cells. Several of the described disorders may terminate in malignancy following a prolonged period of dysregulated proliferation (Table 1).

Hemophagocytic Lymphohistiocytosis

EBV is one of the recognized causes of sporadic hemophagocytic lymphohistiocytosis (HLH), a disorder characterized pathologically by generalized histiocyte proliferation and hemophagocytosis (173). Patients with this unusual syndrome present with fever, generalized lymphadenopathy, hepatosplenomegaly, hepatitis, pancytopenia, and coagulopathy. T-cell proliferation is a primary feature of HLH. The proposed pathogenesis of this disorder suggests that infection of T cells by EBV selectively upregulates the expression of tumor necrosis factor-α (TNF-α), which, in combination with IFN-γ and other cytokines, can activate macrophages. While sporadic HLH is not associated with known immunodeficiency (174), unlike familial HLH in which mutations in perforin and other genes compromise cytotoxic granule delivery, it is likely that a significant subset of patients will eventually fall into this category (175). Therapy typically includes corticosteroids, etoposide, and cyclosporin, but some patients may require HSCT. HLH may be a harbinger of chronic active EBV (see below) or an EBV-bearing T-cell malignancy (176).

TABLE 1 Epstein-Barr Virus–Associated Atypical Lymphoid Hyperplasia

Hemophagocytic lymphohistiocytosis (HLH)
Lymphomatoid granulomatosis (LG)
Lymphocyte interstitial pneumonitis (LIP)
Chronic active EBV (CAEBV)
Some primary immunodeficiency disorders

Lymphomatoid Granulomatosis

Lymphomatoid granulomatosis is an angiodestructive disorder of the lymphoid system; over 90% of cases have been associated with EBV infection (177–179). In the majority of cases, EBV-infected B cells are present, and the B-cell proliferation is clonal. Patients often have evidence of immunodeficiency, including congenital and acquired conditions such as HIV infection. The pathogenesis of this disorder is thus likely directly related to the transformation of EBV-infected B cells in an environment with impaired T-cell function. Patients with lymphomatoid granulomatosis may respond to IFN-α (177). Clinical features include fever, cough, malaise, and weight loss, with involvement of lung, kidney, liver, skin, and subcutaneous tissue and the central nervous system, with the typical histologic changes.

Lymphocytic Interstitial Pneumonitis

Lymphocytic interstitial pneumonitis (LIP) is a lymphoproliferative disorder characterized by lymphocyte-predominant interstitial lung infiltrates, most often involving the bilateral lower lobes. Diffuse infiltration of the interstitium and alveolar spaces by lymphocytes and plasma cells is typical. The main clinical symptoms are gradual onset of dyspnea and cough of approximately 6 months' duration. Less frequently, systemic symptoms may include fever, night sweats, arthralgia, and weight loss. Hypertrophy of the salivary glands is observed in 20% of patients. LIP is causally associated with autoimmune diseases, as well as other virus infections including HIV (primarily pediatric) and human T-lymphotropic virus 1 (HTLV-1). A subset of cases is associated with EBV, and all forms reflect immune dysfunction. The natural history is variable with immunosuppressive agents, particularly corticosteroids sometimes used to treat disease, although success is variable (180–182).

Chronic Active EBV Infection

Chronic active EBV (CAEBV) infection is a rare disorder that is characterized by recurrent fever, lymphadenopathy, and hepatosplenomegaly persisting 6 months or longer (183). CAEBV is accompanied by grossly abnormal EBV antibody titers, with IgG antibody to EBV VCA often in excess of 1:5,000. High EBV viral loads and EBV monoclonality are present in the majority of CAEBV patients. EBV-infected T cells or NK cells circulate in the peripheral blood, particularly of Asian patients with CAEBV, and development of HLH as well as T or NK cell malignancies is frequent. Individuals with EBV-infected T cells had a 5-year survival rate of less than 50%, whereas those with the NK phenotype had a 5-year survival rate of 80%. B-cell disease can also occur, and mutations in perforin and syntaxin binding protein 2 (STXBP2; required for release of cytotoxic granules) have been described in this setting (184). Etoposide-containing therapy may be effective, although HSCT is often required for cure (185).

X-Linked Lymphoproliferative and Other Primary Immunodeficiency Disorders

X-linked lymphoproliferative (XLP1) syndrome-1 (Duncan's syndrome) was the first primary immunodeficiency identified by a selective inability to eliminate EBV-infected B lymphocytes (186), although in fact elimination of all activated B cells is compromised in XLP1 (187). XLP1 is manifested by severe or fatal AIM, acquired immunodeficiency, and lymphoma development (186). The gene (SH2D1A) responsible for XLP1 syndrome encodes a 128-amino-acid protein

that binds the cytoplasmic domain of the signaling lymphocyte activation molecule (SLAM) family of surface receptors on hematopoietic cells called SLAM-associated protein (SAP). The SLAM family proteins transduce activating signals that facilitate cell-cell cross talk and require SAP functions to do so (188). During acute EBV infection, males with XLP1 develop vigorous EBV-directed T-cell activation. However, in the absence of SAP, B-T/NK cell conjugates are unstable, T cells show poor effector function, and local responses fail to resolve (150). While acute therapy for XLP1 syndrome is often transiently directed to eliminate EBV-transformed B lymphocytes (Rituximab), definitive treatment of XLP1 syndrome currently requires HSCT, which confers an 80% 1-year survival. Following discovery of the mutation causing XLP1, a subset of other congenital mutations that alter clearance of EBV-bearing cells by T and NK cells has been uncovered. However, in many more cases, EBV represents but one among a broader spectrum of altered pathogen susceptibilities (151).

The second X-linked syndrome (XLP2), with manifestations overlapping those of XLP1, involves mutation of the gene encoding the X-linked inhibitor of apoptosis protein (XIAP). XLP2 T cells are highly susceptible to apoptosis from various stimuli, including T-cell restimulation; thus, effector functions are poor. EBV infection results in hepatosplenomegaly, HLH, and hypogammaglobulinemia. Notably, XLP2 males do not develop lymphoma, but do develop colitis, although the mechanism remains unknown (189).

A third X-linked immunodeficiency disease, XMEN (X-linked immunodeficiency with magnesium defect, EBV infection, and neoplasia) results from mutation of MAGT1, which encodes a magnesium transporter on T cells that transduces signals required for T-cell receptor stimulation. Poor responses to other herpesvirus infections and even bacterial infections have been detected in XMEN patients.

In contrast, CD27 deficiency (a TNF receptor family member and marker of B- and T-cell memory) is caused by an autosomal recessive mutation. CD27 deficiency is highly associated with a spectrum of diseases focused on EBV. Mutations in ITK, CORO1A, and LRBA are primarily, but not exclusively, linked to EBV-related pathology. Mutations in CD16, MCM4, GATA2, and LYST that diminish NK cell responses as well in PIK3CD, PIK3R1, CTPS1, STK4, FCGR3A, CARD11, ATM, WAS, and SCIDs, although often first identified in the setting of progressive EBV disease, in fact cause defective clearance of multiple pathogens (reviewed in 153).

EBV-Associated Malignancies

Distinction between the lymphoproliferative disorders (LPDs) associated with EBV infection and the EBV-bearing lymphoid malignancies that frequently result can be blurred. In virtually every instance, some form of T- or NK-cell compromise is contributory. In the absence of congenital immunodeficiency, a major environmental insult (e.g., chronic infection, therapy for cancer or autoimmune disease) to the immune system is often, although not always, identified (Table 2 and Table 3). With developments in advanced sequencing, personalized medicine, biologics, and immune-based therapies, the approach to treatment of individual cancers is in flux; therefore, further review for each tumor type is recommended.

Posttransplant Lymphoproliferative Disorders

EBV is associated with LPDs in transplant patients—both HSCT and SOT recipients on immunosuppressive therapy (190). Posttransplant LPDs range from polyclonal B-cell proliferations (type III latency) to malignant monoclonal B-cell lymphomas (type II). EBV-positive T-cell LPD occurs, but is rare. Polyclonal LPD is most common during the first year after HSCT when immunosuppression is maximal. EBV-positive recipients who receive HSCT from donors who are EBV-negative are at highest risk due to rapid infection of the new immune system in the absence of functional T cells. Systemic symptoms of fever and sore throat may be evident on presentation. These symptoms and lymphadenopathy will sometimes respond to reduction in immunosuppressive therapy alone. Anti-CD20 monoclonal antibody therapy (e.g., Rituximab) that depletes B cells is often employed,

TABLE 2 Diverse Forms of Cellular Immunodeficiency (T, NK) That Predispose to Epstein-Barr Virus Disease

Coinfection (chronic and persistent)
HIV/AIDS
Malaria
Iatrogenic (therapy related)
Transplant recipients
Autoimmune disease
Cancer chemotherapy
Select primary immunodeficiency diseases
Some autoimmune diseases
Extremes of age
Very young
Very old

HIV, human immunodeficiency virus; AIDS, acquired immunodeficiency syndrome.

TABLE 3 Spectrum of Epstein-Barr Virus–Associated Disease

Immunocompromised host	"Normal host"
Primary infection	
Runaway infectious mononucleosis	Asymptomatic infection
Hemophagocytic syndrome	AIM
Reactivated infection	
B-PTLD	B-NHL (elderly)
B-NHL, various	
Burkitt's lymphoma	Endemic Burkitt's lymphoma
Hodgkin's lymphoma	Hodgkin's lymphoma
T-LPD (rare)	T/NK-cell lymphoma
Chronic active EBV	Monocytoid (rare) tumor
Leiomyosarcoma	
Oral hairy leukoplakia	Nasopharyngeal carcinoma
	Lymphoepitheliomas, various
	Gastric adenocarcinoma
	Other epithelial cell carcinomas, subset of head and neck, breast?
Autoimmune diseases: MS, SLE	

AIM, acute infectious mononucleosis; B-PTLD, B-cell posttransplant lymphoproliferative disease; B-NHL, B-cell non-Hodgkin's lymphoma; T-LPD, T-cell lymphoproliferative disease; EBV, Epstein-Barr virus; NK, natural killer; MS, multiple sclerosis; SLE, systemic lupus erythematosus.

although it increases the vulnerability of posttransplant recipients to infection (191). Many centers now monitor EBV load in the circulation early after transplant by PCR. Optimized detection methods are in development (192, 193). High or rising loads predict (but do not prove) incipient disease. If immunosuppression cannot be reduced or is ineffective, prophylactic Rituximab is often administered (194). Anecdotal use of other agents such as interleukin 2, IFN-α, and intravenous Igs has been reported. No clear-cut benefits of such modalities have been demonstrated. Prophylactic ganciclovir, administered to prevent CMV disease early after transplant, can effectively reduce lytic replication of EBV and thus may limit new B-cell infection, potentially decreasing early disease, but systematic validation is needed (195, 196). EBV-specific T cells have been employed successfully both prophylactically and therapeutically for treatment of PTLD with most success in HSCT recipients (see below).

The frequency of LPD following receipt of a solid organ allograft is related to the degree, type, and duration of immunosuppression and history of infection. Reported rates of LPD for children and adults following receipt of different allografts are given in Table 4. In contrast to HSCT, an EBV-seronegative recipient who receives an EBV-positive allograft is at greatest risk of disease, similar to CMV infection (197). Pediatric recipients of SOT who develop primary infection after transplantation have an extremely high mortality rate compared with previously infected children (198), underscoring the need for vaccine development. Onset of a monoclonal B-cell lymphoma may be preceded by benign polyclonal B-cell proliferation or present with sudden growth of a solid tumor mass in the organ allograft or any other tissue.

These lymphomas may be polymorphic in appearance, although the tumor mass is usually monoclonal and gene expression patterns often resemble those of diffuse large B-cell lymphomas (DLBCL). Because SOT recipients maintained on long-term immunosuppressive therapy are more likely to develop monoclonal disease, steps to minimize immunosuppressive therapy over time should be a priority (199). Late-onset disease is often resistant to treatment and the clinical course is usually the same as for an aggressive lymphoma. If chemotherapy [e.g., cyclophosphamide, doxorubicin (hydroxydaunomycin), vincristine (Oncovin®), and prednisolone (CHOP) or other] plus Rituximab is ineffective, cell-based therapies, including EBV-specific T-cell lines (EBVSTs), are being used increasingly (190). They are labor intensive, costly, and can sometimes exacerbate graft-versus-host disease, but can be effective (200). T-cell checkpoint inhibitors in the form of monoclonal antibodies that block the interactions between surface antigens such as PD-L1 on tumors and PD-1 on T cells (as well as other checkpoint inhibitors) show promise for future treatment of EBV-associated LPD in combination with cell-based approaches (201).

TABLE 4 Rates of Posttransplant Lymphoproliferative Disease among Solid Organ Transplant Patients

	Pediatric		Adult	
Organ	1 year	5 years	1 year	5 years
Lung/heart-lung	4.0%	16%	1.0%	1.5%
Liver	2.1%	4.7%	0.25%	1.1%
Pancreas (isolated)	N/A	N/A	2.3%	2.3%
Heart	1.6%	5.7%	0.3%	0.7%
Kidney	1.3%	2.4%	<0.2%	0.6%

Cumulative 1- and 5-year incidence of PTLD in pediatric and adult SOT recipients by transplanted organ as reported in the 2010 Organ Procurement and Transplant Network (OPTN)/Scientific Registry of Transplant Recipients (SRTR) Annual Report. Data for intestinal transplant recipients not broken down by pediatric versus adult and therefore not included.

PTLD, Posttransplant lymphoproliferative disease; SOT, solid organ transplant; N/A, not available.

EBV-Associated Malignancies in HIV Infection

In the setting of profound immunodeficiency associated with HIV infections, non-Hodgkin's lymphomas (NHLs) have been observed to occur approximately 60- to 100-fold more frequently than expected (202). A study conducted from 1984 to 1992 [before combination antiretroviral therapy (cART)] showed that EBV was associated with 39 of 59 (66%) of HIV-related systemic lymphomas (203). Analysis of EBV terminal repeats in these lymphomas again confirmed their monoclonal origin, and *c-myc* rearrangements were noted in up to 40%, indicating a Burkitt-like pathogenesis. B-LPD-like disease was most prevalent among patients with frank acquired immunodeficiency syndrome (AIDS), whereas HL was less common (discussed below). For unknown reasons, many EBV-associated NHLs in persons with HIV present with primary central nervous system lymphomas (204). Whereas B-LPD-like disease has virtually disappeared in the post-cART area, certain NHLs and HL, although decreased in HIV-infected individuals successfully treated with cART, nevertheless remain elevated relative to the general population (205).

A less serious EBV-induced disease in persons with AIDS is oral hairy leukoplakia, an unusual geographic, gray wart-like lesion of the lingual squamous epithelium. Virus replication is evident only in the upper layers of the epithelium and is effectively inhibited by nucleoside analogs, such as acyclovir or ganciclovir. Oral hairy leukoplakia lesions, initially thought to be specific for HIV-related immunodeficiency, have now been observed in other immunosuppressed patients and rarely in healthy individuals (206).

EBV and Smooth-Muscle Tumors

Children and young adults with AIDS experience an unusually high incidence of smooth-muscle tumors (leiomyomas and leiomyosarcomas) (207). These tumors are often multifocal and can also localize to the central nervous system (208). Convincing evidence for an etiologic role of EBV in the development of these neoplastic lesions was provided by demonstration that these smooth-muscle tumors contain clonal EBV and also develop with increased frequency in transplant recipients (209).

Primary Effusion Lymphoma

Primary effusion lymphoma (PEL) is an uncommon B-cell malignancy that occurs almost exclusively in the T-cell compromised host (e.g., AIDS and transplant patients). It is characterized by lymphomatous effusions in pleural, peritoneal, and other body cavities and is comprised of malignant postgerminal center type B cells. PEL is an aggressive disease with inflammatory-type symptoms and very poor prognosis. While Kaposi's sarcoma herpesvirus (KSHV, HHV-8) is always present in PEL, 70% to 80% of these tumors are co-infected with EBV (latency I). Studies suggest EBV coinfection provides a survival advantage *in vivo* because coinfected cell lines are more tumorigenic in small-animal disease models (210). There is no standard therapy for PEL at this time (211).

Hodgkin Lymphoma

EBV genomic DNA was first reported in HL in 1987. Evidence supports a role for EBV in the pathogenesis of classical HL because malignant Reed-Sternberg cells contain the EBV genome in up to 50% of "Western" cases (212). Furthermore, AIM is a risk factor for subsequent development of classical HD (nodular sclerosing, mixed cellularity) that is variably reported as 3- to 10-fold increased during the 10-year period after AIM. The basis for this association remains unknown, but is supported by studies in countries such as Singapore, where rapid development has led to an increase of both diseases in young adults (108). As with BL (see below), detection of EBV in Reed-Sternberg cells appears to vary geographically and by age; for example, 94% of classical HD (mixed cellularity) cases occurring in Peru contain EBV transcripts (213) and globally almost all childhood cases of HD are EBV positive (214). Recently, a risk locus for EBV-bearing HL in European populations was described on chromosome 6p21.3 (215). The pattern of EBV latency in HD resembles type II latency as observed in NPC. Both are characterized by rich cellular infiltrates. EBNA-1 is expressed from the Qp promoter, whereas expression of other EBNAs is prevented by the silent Cp and Wp promoters. LMP-2, LMP-1, EBER transcripts, and BART miRNAs are detected irrespective of histologic subtype (216). The EBV genome is also usually present in cases of HL (mixed cellularity) that appear in individuals with primary immunodeficiency or AIDS (217). Among persons successfully treated with cART, the incidence of LPD-like tumors has greatly diminished and other NHLs are somewhat diminished, whereas the incidence of HL has remained high for reasons that are not well understood (216, 218).

Burkitt's Lymphoma

BL is among the most common childhood malignancies in equatorial Africa (219, 220). This unmistakable tumor is typically localized to the jaw of young patients and occurs more frequently in males, as do all EBV-associated malignancies. The majority of endemic cases occur in discrete geographic climates located along the *P. falciparum* malaria belt across Africa. Analysis of the EBV genome terminal-repeat copy number in endemic BLs showed that tumors originate from a single EBV-infected B cell. Products derived from recurrent bouts of *P. falciparum* malaria during early childhood (e.g., Pfemp1, hemazoin, others) are believed to provide a chronic stimulus for proliferation and differentiation of B lymphocytes (221, 222) that already carry high loads of latent EBV because of T-cell exhaustion resulting from chronic malaria infection (223). As these B cells differentiate, expression of activation-induced cytidine deaminase (AID) in the course of the germinal center reaction facilitates *c-myc* translocation and multistep tumorigenesis (224).

Tumor cells from areas where BL is endemic virtually always contain copies of the EBV genome in contrast to tumor cells from patients with sporadic cases of BL from areas of low incidence (>95% versus 20%). BL-like tumors are increased in HIV-infected persons, even on highly active antiretroviral therapy (HAART) (40% to 50% EBV positive) (225). The malignant cells obtained from fresh tumor biopsy samples consistently display a homogeneous surface phenotype, including the pan-B-cell marker (CD20), the common acute lymphoblastic leukemia antigen (CALLA, CD10), and the BL-associated antigen (CD77), consistent with a germinal center origin. These cells do not express the common B-cell activation antigens CD23, CD30, CD39, and CD70 or the cell adhesion molecules LFA-1 (CD11a/18), ICAM-1 (CD54), and LFA-3 (CD58) in contrast to the malignant B cells that characterize LPDs and variably certain NHLs. Fresh BL tumor cells retain a resting B-cell phenotype and typically express only EBNA-1, EBERs, and ncRNAs. It is speculated that eradication of *P. falciparum* malaria will result in eradication of endemic BL, but studies to date are inconclusive (220). In cases of sporadic BL and classical HL that lack EBV genomes, the specter of a transient EBV infection has been raised, but has not been proven to date.

T-Cell and NK-Cell Lymphomas

Although much less common than B-cell infection, normal activated T-cell populations can variably express the EBV attachment receptors CD21 and CD35 as well as HLA class II, providing a local environment permissive for EBV entry (226, 227). Supporting this observation, studies by Anagnostopoulos et al. demonstrated EBV-infected tonsillar T lymphocytes in individuals with AIM (120). The events that determine whether T-cell latency or immortalization results are unknown. EBV-positive T- and NK-cell lymphomas, though rare, occur in several settings. EBV T-cell LPD is sometimes diagnosed following iatrogenic immunosuppression as required for transplant, similar to B-LPD (228). Individuals with CAEBV infection often succumb to EBV-bearing T-cell lymphomas (229). Two major forms of primary EBV-associated T-cell lymphoma display distinct geographical distributions. Both are aggressive tumors, poorly responsive to chemotherapy, and both occur most frequently in middle-aged men. Peripheral T-cell lymphomas not otherwise specified (PTCL-NOS) are ∽40% EBV positive and are primarily encountered in North America and Europe. In contrast, extranodal NK/T-cell lymphomas, nasal type (ENKT), the most common form of extranodal lymphoma worldwide, are prevalent in Asia and South and Central America. Similar to several EBV-associated malignancies, a rich lymphoid infiltrate is present in these tumors. The molecular biology of T-cell tumors is currently poorly understood. Reports of viral gene expression include EBERs, EBNA-1, and LMP-1 (more variably), most consistent with type I/II latency (230).

Nasopharyngeal Carcinoma

Worldwide, nonkeratinizing NPC is relatively rare; however, it is one of the most common cancers in southern China, with age-adjusted incidence rates of up to 25 to 100 per 100,000 (231). Middle-aged men are most frequently affected. The incidence of undifferentiated NPC is also increased throughout Southeast Asia, among Alaskan natives, and around the Mediterranean, with North African men typically diagnosed at younger ages than Asian patients. Genetic predisposition and environment are recognized as contributory factors. In contrast to BL, EBV is present in every anaplastic NPC cell regardless of geography. The presence of a single clonal form of EBV in premalignant lesions, such as nasopharyngeal dysplasia or carcinoma *in situ*, indicates that EBV-induced cellular proliferation precedes the acquisition of full invasiveness. EBERs and EBNA-1 are universally detected, whereas LMP-1 and LMP-2 are detected in most tumors as are ncRNAs (latency II). Although EBNA-2 is essential for the initial transformation of lymphocytes, its absence in preinvasive neoplasia in NPC indicates that EBNA-2 is not required for the altered epithelial cell growth associated with NPC (232, 233). Radiation has long been a major component of treatment together

with chemotherapy; new potential therapeutic targets are emerging (234).

Gastric Carcinoma

EBV-associated gastric carcinoma was first reported by Burke et al. in 1990. The EBV-bearing tumors that contain a rich lymphoid infiltrate similar to NPC form part of the spectrum of EBV-associated lymphoepithelial carcinomas (NPC, thymoepitheliomas, salivary gland tumors), whereas the gastric adenocarcinomas are distinct (235). The distribution of the latter appears to be worldwide, with young rather than middle-aged men primarily affected. Although only ∽10% of all gastric cancers contain EBV, because gastric cancer is the second leading cause of cancer deaths worldwide, EBV-bearing gastric carcinoma is potentially the most common of all EBV-related malignancies. Current treatment is the same as for other forms of gastric cancer. Complete surgical resection is especially effective because the EBV-associated adenocarcinomas do not readily metastasize to lymph nodes and carry an improved prognosis compared with EBV-negative forms of gastric cancer. Viral gene expression is most consistent with a latency I/II program, although expression of limited lytic cycle proteins, such as the immunoevasin BNLF2a has also been detected (236, 237).

Other EBV-Infected Tumors

There are limited reports of atypical tumor types sometimes bearing EBV genomes, primarily tumors of other hematopoietic and epithelial lineages. In some cases, detection of contamination by EBV-positive B cells in the tumor microenvironment has been a concern, especially when PCR alone was applied for detection. In others, more extensive analyses complemented by immunohistochemistry, *in situ* hybridization, and other technologies (238) have provided more compelling evidence that EBV is present in these cancer cells and may have contributed to multistep tumorigenesis, perhaps following aberrant expression of virus entry proteins by a premalignant host cell in a microenvironment where EBV load is serendipitously high.

EBV and Autoimmune Disease

Genetic predisposition combined with an environmental trigger(s) can result in a spectrum of diseases manifested by immune dysregulation and pathologic targeting of self. Similar to patients with AIM, who often manifest transient autoimmunity, patients with systemic autoimmune disease often demonstrate elevated antibody levels to VCA and EA as well as elevated titers of EBV DNA in blood, consistent with altered T-cell function. Although controversy persists (239), more definitive epidemiologic and clinical data support EBV as a major environmental trigger for MS (240) and possibly SLE. In the case of MS: (1) virtually all patients, including very young children, are EBV seropositive; (2) development of AIM almost always precedes MS; and (3) elevated IgG responses to a distinct subdomain in EBNA-1 similar to myelin, but not to most other EBV antigens, occurs years before MS becomes clinically apparent, supporting molecular mimicry as a disease mechanism (241). In contrast, AIM does not predict lupus development, and the spectrum of autoantibodies is distinct and broader (242, 243). However, among young children that develop SLE the seroprevalence of EBV is much higher (99%) than that of closely matched peers (70%). Whether EBV contributes to the pathogenesis of rheumatoid arthritis (244), Sjögren's syndrome (245), or other autoimmune diseases is under investigation in several laboratories.

LABORATORY DIAGNOSIS

Primary Infection

To establish a diagnosis of AIM among persons with clinically suspected disease, evaluation of the white blood cell count can be especially useful, as lymphocytosis with >10% atypical lymphocytes is often present before either heterophile or IgM antibodies to VCA, conventional criteria for confirming new infection, can be detected. Total lymphocytosis of >50%, the presence of monocytosis, together with >10% increasing to >40% atypical lymphocytes in combination virtually assures a diagnosis of AIM, although relatively few patients will meet all of these conditions (166). (See also the section on Humoral Immune Response for additional discussion.)

The classic serologic response that is used to diagnose primary EBV infection in clinical practice is depicted in Fig. 6 (above), although variation often occurs. IgM to VCA, predicted to arise at the time of clinical illness, may be delayed by several days and may first appear together with IgG to VCA. If IgM VCA is negative and suspicion of primary infection is high, retesting is warranted. IgM antibodies to VCA (also heterophile) disappear within 2–6 months and failure to do so suggests a process other than primary EBV infection should be suspected. Antibody to the EA complex rises at the time of clinical presentation and then, like IgM antibody, disappears after several months. Both may reappear in the setting of poorly controlled lytic reactivation associated with T-cell compromise and malignancy. Production of antibodies to the nuclear antigen complex EBNA is delayed by several months following primary infection. Thus, detection upon initial evaluation indicates that the clinical syndrome is not primary. One caveat is that some individuals never develop anti-EBNA antibodies.

Although primary infection is usually accompanied by viremia, EBV PCR is not useful in healthy individuals because intercurrent viremia may occur among persistently infected individuals. On the other hand, EBV PCR can be an important tool for diagnosing primary infection in the immunocompromised host who is known to be EBV negative and may fail to mount a detectable or conventional antibody response upon primary infection. In many centers, EBV-negative transplant recipients (testing is performed pretransplant) are surveyed by EBV PCR at defined intervals to identify a new infection and implement strategies to control disease.

Reactivation

Although rising antibody titers to EBV lytic-cycle antigens appear to reflect loss of cellular immune control relevant to several EBV-associated diseases, they have been primarily used to (1) establish a diagnosis of CAEBV (high titers of IgG to VCA) and (2) diagnose and treat NPC (high or rising titers of IgA to VCA). Determination of viral load by PCR has now been validated as a biomarker of incipient or established malignant disease in multiple settings, although optimal methods remain under investigation (192). Changes in viral load have been used to implement preemptive strategies to diminish the risk of PTLD as well as to follow the response to treatment of the various EBV-associated tumors.

PREVENTION

Immunization

Evidence implicating EBV in the etiology of a variety of human diseases has made the prospect of developing a

virus-based vaccine very appealing. Vaccine administration in resource-rich countries and increasingly on a global basis would prevent AIM in young adults. With the annual incidence of AIM estimated in the United States at 100,000 cases, EBV causes significantly more illness than mumps, for which a successful vaccination strategy exists.

As discussed, gp350 mediates B-cell attachment. It is one of the most abundant late viral proteins present in lytically infected cell plasma membranes and is the most abundant protein on the outer surface of the virus coat. Passively transferred antibodies are likely important in protecting infants from EBV infection. As most of the human EBV-neutralizing antibody response is directed against gp350 (130), gp350 has been the major EBV lytic-cycle protein targeted for subunit vaccine development. The largest study, a phase II, randomized, double-blind, placebo-controlled trial of an adjuvanted recombinant gp350 vaccine was undertaken in healthy, EBV-seronegative young adults (246). More than 90% of vaccinated individuals developed anti-gp350 antibodies. Importantly, the vaccine demonstrated efficacy (78%) in preventing symptomatic primary EBV infection (AIM), but was not effective in preventing asymptomatic infection. These data justify development and testing of anti-gp350 vaccines (247) such as virus-like particle vaccines (248) and others that incorporate gp350 (as well as other novel candidates) and also recruit T cells. Although development of potent neutralizing antibody responses is important, given that $CD8^+$ T-cell responses are essential for controlling EBV replication and LPD, future vaccine research defining additional protective T-cell epitopes for inclusion in EBV vaccines will also be key (249).

Prophylactic Therapy

Antivirals have been administered transiently to prevent primary EBV infection, particularly in seronegative children who are at high risk of PTLD following an EBV-positive SOT; however, antivirals will not prevent tumors that arise from latently infected donor B cells. IVIG or CMVIG, which contains high-titer anti-EBV antibodies, has been used with unproven benefit to prevent primary EBV infection (250, 251). Administration of monoclonal antibodies, such as anti-CD20 (e.g., Rituximab) can abort expansion of EBV-positive B cells en route to PTLD but compromise humoral responses to new infections. Prophylactic treatment of HSCT recipients at high risk of PTLD with EBV-specific T cells (EBVST) has been successful in some settings. These varied approaches to prophylaxis and treatment are discussed in more detail below.

TREATMENT

Symptomatic and Anti-Inflammatory

The mainstay of treatment for individuals with AIM is supportive care. Acetaminophen or nonsteroidal anti-inflammatory agents are recommended for the treatment of fever, throat discomfort, and malaise. Provision of adequate fluids and nutrition is also appropriate. Although ensuring adequate rest is prudent, bed rest *per se* is unnecessary. Contact sports should be avoided due to the risk of splenic rupture.

The use of corticosteroids in the treatment of EBV-induced AIM remains controversial. Studies looking at the use of corticosteroids have been imperfect, but they do suggest that these agents induce a modest improvement with reduction of lymphoid and mucosal swelling. Thus, a trial of corticosteroids in individuals with impending airway obstruction (manifested clinically by difficulty breathing in the recumbent position) is warranted. In addition, individuals suffering from severe, overwhelming, life-threatening infection (e.g., liver failure), along with individuals who sustain other severe complications such as aplastic anemia, should also be considered for corticosteroid therapy, although data supporting benefits in these situations, because of their rarity, are lacking.

Despite the recommendations of some experienced clinicians that corticosteroids be administered in routine cases of AIM, the literature does not support this approach. The clinical illness of AIM represents the immune response to EBV, an agent that establishes lifelong latency and that has oncogenic potential. For this reason, the administration of immunomodulating agents such as corticosteroids during primary infection is theoretically contraindicated because of the possibility of altering the immune response and predisposing the patient to a long-term lymphoproliferative complication. Indeed, studies of individuals with AIM who received corticosteroids many weeks earlier have demonstrated globally diminished numbers of B cells and T cells, including diminished numbers of CD4 T-helper and CD8 T cytotoxic cells (252). Since no long-term data obtained for individuals who received corticosteroids during primary EBV infection are available, it would seem prudent, despite the potential of short-term improvement, to withhold such treatment from most individuals given the self-limited nature of this infection in the vast majority of cases (171).

Antiviral Treatment

Acyclovir and ganciclovir are nucleoside analogs that can inhibit lytic EBV replication following phosphorylation by the EBV phosphotransferase (BGLF4) (253). This enables incorporation of their triphosphate form into viral DNA by the EBV DNA polymerase and is followed by DNA chain termination (replication arrest). These agents have no effect on latent infection. Specific therapy of acute EBV infections with intravenous and oral formulations of acyclovir has been studied (254, 255). Short-term suppression of viral shedding can be demonstrated, but significant clinical benefit has not been demonstrated. These results are not surprising in view of data documenting that viral load has likely peaked at the time of presentation with AIM and that the manifestations of AIM are more likely due to aberrant immune responses (99). Long-term administration of valacyclovir over 1 year diminished the frequency of EBV-infected B cells in normal volunteers. On the basis of this study, it was estimated that if possible, it would take 11.3 years of continuous treatment to eradicate EBV (256).

In the majority of the EBV-associated malignancies, where the stage of the virus infection cycle has been characterized, there is little evidence for permissive (lytic) infection. Standard chemotherapy that varies based on tumor type and not specifically directed at the virus is still employed for most EBV-associated tumors discussed in the above section. Because nucleoside analogs that inhibit polymerase function (acyclovir, ganciclovir, cidofovir, foscarnet, and their congeners) are only effective in inhibiting replication of linear EBV DNA, there is little to be gained by their use as single agents in diseases associated with latent infection. There is anecdotal support for their use in EBV-induced histiocytic hemophagocytosis (acyclovir), where evidence of replicating EBV has been demonstrated (257). Definitive studies regarding prophylactic use in the immunocompromised host, such as children who are EBV seronegative at the time of SOT and are at risk of severe primary infection and

subsequent PTLD, are lacking (196). However, routine prophylactic use of the aforementioned nucleoside analogs to prevent CMV and HSV in the peritransplant period may also diminish the risk of primary EBV infection and PTLD. Measurements of EBV viral load in the circulation reflect both lytic replication and outgrowth of latently infected cells; thus, if the titer of circulating virus is decreased, the likelihood of additional B-cell infection and posttransplant lymphoproliferative disease (PTLD) may also be decreased. Robust lytic cycle induction of latently infected tumor cells—"lytic induction therapy"—remains an area of active research, and, if successful, would potentially expand a role for both antivirals and novel cell-based therapies (258). Small-molecule inhibitors that target key viral as well as cellular proteins are also under investigation (259).

Cell-Based Therapies

The presence of viral proteins in EBV-associated tumors has provided an impetus for development of T-cell-based immunotherapies crafted with increasing sophistication over the years (260, 261). Although labor intensive, expensive, and only available in limited cancer centers, the tide is beginning to turn due to increasing support of cell-based therapies by the biotechnology sector. To date, the greatest success of EBVSTs has been achieved upon treatment of PTLD in HSCT recipients, as viral protein expression is robust (type III latency) creating multiple potential T-cell targets and decreased cellularity has facilitated outgrowth and function of infused T cells. Although use of unselected donor lymphocyte infusions (DLIs) was often accompanied by side effects, including graft-versus-host disease and cytokine release syndromes, EBVSTs have proved far more efficacious and devoid of most toxicity. Therapy of SOT patients has proved more challenging because of potential HLA mismatch, diminished viral protein expression in most tumors, and the requirement for ongoing immunosuppression reducing the efficacy of infused T cells. However, banks of EBVSTs have now been developed that are able to provide a rapid source of cells with partial HLA matches that have been used with some success. Tumors in which viral gene expression is comparatively reduced (type II latency, e.g., NPC, HL, some NHLs) have responded less well to EBVSTs, but there is optimism that the introduction of checkpoint inhibitors together with EBVSTs will improve outcomes. Type I latency-expressing tumors have not been assessed systematically at this time. The introduction of T-cells activated with chimeric antigen receptor (CAR; often a monoclonal antibody directed to a specific cell type) into clinical practice suggests that a combination strategy could result in enhanced tumor cell localization of EBVSTs increasing future efficacy (262, 263).

Therapeutic Immunization

Although the safety bar is set very high for a prophylactic vaccine, therapeutic vaccine candidates that incorporate EBV products expressed by EBV-associated malignancies are also under development using vectors optimized to stimulate a vigorous cellular immune response capable of eradicating established tumors (249). Current targets include, in particular, NPC and gastric carcinoma with some promising early-phase results.

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Kaposi's Sarcoma-Associated Herpesvirus (KSHV/HHV8)

YUAN CHANG, SHOU-JIANG GAO, AND PATRICK S. MOORE

26

Discovered in 1994, Kaposi's sarcoma-associated herpesvirus (KSHV or HHV8) causes several human cancers, including Kaposi's sarcoma (KS), primary effusion lymphomas (PEL), and some forms of multicentric Castleman's disease (MCD). KSHV is commonly associated with cancers among AIDS patients but it is also a significant public health problem in developing countries for both HIV-infected and uninfected populations. KSHV is a gammaherpesvirus with unique features in its gene products, gene distribution and evolution, and mechanisms of cellular transformation. The epidemiology of KS in different risk groups and geographic regions parallels the prevalence of KSHV infection. While modes of transmission of KSHV are still to be fully determined, specific measures can be implemented to prevent its spread. Specific assays, including serologic and antigen immunohistochemistry tests, have been developed that allow detection of infected patients and patient tissues. Understanding of the molecular mechanisms for KSHV-related pathogenesis should facilitate the detection, prevention, and therapy of KSHV and its associated cancers.

INTRODUCTION

Kaposi's sarcoma-associated herpesvirus (KSHV; also called human herpesvirus-8 [HHV-8]) is a large double-stranded DNA herpesvirus that is the cause of Kaposi's sarcoma (KS) and primary effusion lymphomas (PEL). KSHV is also the etiologic agent in the majority of multicentric Castleman's disease (MCD). KSHV belongs to the Rhadinovirus genus of the gammaherpesvirus subfamily. Similar to other herpesviruses, KSHV has both latent and lytic replication cycles, and infection is presumed to be lifelong. KSHV has evolved sophisticated mechanisms at multiple levels to facilitate its infection and replication, resulting in maintenance of persistent infection in immunocompetent hosts and induction of disease, predominantly in immunocompromised hosts.

KS, the most common malignancy associated with KSHV, has a dynamic and evolving epidemiologic profile. A rare cancer in the Western world prior to the emergence of HIV, the onset of the AIDS epidemic resulted in an explosive increase in the incidence of KS. With the success of antiretroviral therapy (ART), KS rates have declined but it remains a significant problem with distinct clinical manifestations (1). In Africa, where it has long been a highly prevalent cancer, KS is now the most common cancer in several regions (2), in large part because of the AIDS pandemic in populations lacking effective preventive and therapeutic intervention.

KS, a spindle cell tumor arising from lymphatic endothelium, was first described in 1872 by the Austrian dermatologist and dermatopathologist Moriz Kaposi (3). An infectious agent had been suspected as the cause of the tumor since the 1950s (4). This was reinforced by the emergence of KS as an AIDS-related tumor and its differential occurrence among different HIV risk groups (5). KSHV was discovered in 1994 through a PCR-based DNA subtractive hybridization method in AIDS-KS tissues (6). Shortly after the initial description of KSHV, all forms of KS, in both HIV-positive and HIV-negative patients, were found to harbor the virus. The virus was also found to be B-lymphocyte tropic by its identification in PEL and MCD (7), and cell lines from PELs have become a source for growing the virus in culture (8–10). KSHV was sequenced in 1996 (11) and shown to possess a remarkable array of viral genes homologous to cellular regulatory genes.

VIROLOGY

Classification

KSHV belongs to the genus *Rhadinoviridae* (gamma-2 herpesvirus) of the subfamily *Gammaherpesvirinae* of the family of *Herpesviridae* (12). Among the human herpesviruses, KSHV is most closely related to Epstein-Barr virus (EBV or HHV4), a member of the genus *Lymphocryptovirus* (gamma-1 herpesvirus) (Figure 1). Although *Gammaherpesvirinae* has members broadly represented in mammals, KSHV and EBV are notable as the sole human members of this subfamily, and both are uniquely recognized among human herpesviruses for their tropism for B lymphocytes and their ability to cause human cancer.

Evolution and Genotypes

Early searches for KSHV-like viruses among primates revealed several surprises: KSHV-like rhadinoviruses were discovered to infect rhesus macaques (13) and similar viruses were later discovered among higher primates (Figure 2). All Old World and New World primates appear to be infected with KSHV-like rhadinoviruses (RV-1) that have coevolved

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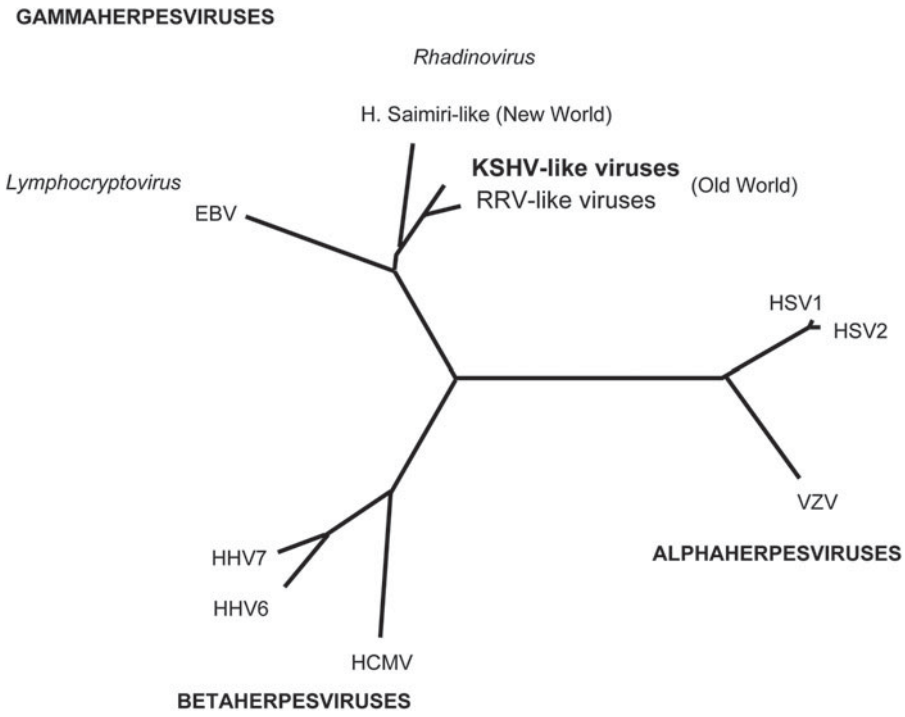

FIGURE 1 A phylogenetic tree showing the alpha-, beta- and gammaherpesvirus subfamilies. KSHV belongs to the genus *Rhadinovirus*, also known as γ-2 herpesviruses, in the lymphotrophic gammaherpesvirus subfamily. Other related gammaherpesviruses are also associated with lymphoproliferative disorders, including EBV in humans and herpesvirus saimiri (HVS) in New World monkeys. Herpesviruses from the alphaherpesvirus (e.g., herpes simplex and varicella-zoster virus) and betaherpesvirus subfamilies (e.g., cytomegalovirus and human herpesviruses 6 and 7) have not been found to cause tumors in humans. (From Moore et al. (12) with permission.)

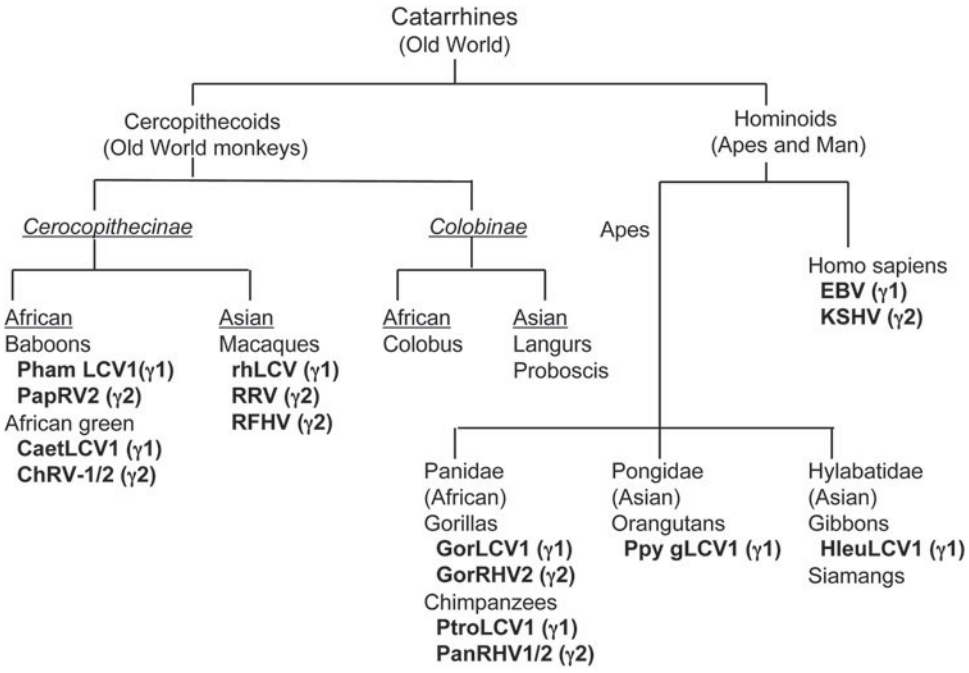

FIGURE 2 Old World primate hosts and their gammaherpesviruses. It is evident from this phylogenetic tree that these are ancient viruses that have coevolved with their hosts. A second rhadinovirus, rhesus rhadinovirus, has been found widely distributed among primates including chimpanzees. It is likely that a human version of this virus exists but has not yet been found. (Courtesy of B. Damania, University of North Carolina, Chapel Hill.)

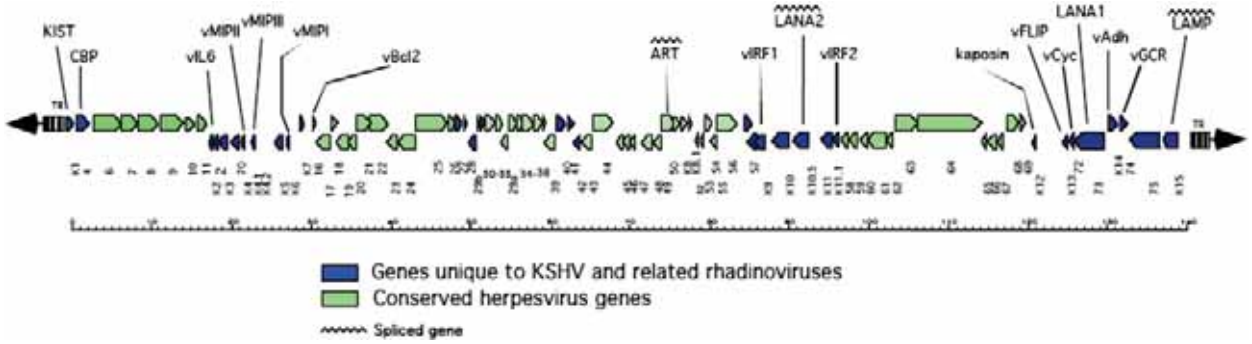

FIGURE 3 Genomic map of KSHV sequenced from the BC-1 KSHV-infected PEL cell line. The viral genome contains a ~145 kb long unique coding region (LUR) flanked on both sides by reiterated terminal repeat (TR) units of high GC content (>85%). Conserved herpesvirus gene blocks (dark blue) are interspersed with blocks containing genes unique to KSHV and other Rhadinoviruses (light blue). These nonconserved regions contain numerous homologs to host cell genes involved in cell-cycle regulation, apoptosis, and immune regulation.

with their hosts. During this search, however, a second distinct but closely related rhadinovirus lineage (RV-2) was found. Members of this lineage include rhesus rhadinovirus (RRV) (14), and pig-tailed macaque RV2 rhadinovirus (MneRV2). These viruses have similar genomic structures and coding genes (15–17). Viruses of this lineage were also found among higher primates (18), leading to the intriguing possibility that there may be an undiscovered RRV ("HHV-9") infecting and possibly causing disease among humans.

KSHV genotyping has been useful in characterizing KSHV transmission (19), and a strong correlation exists between virus evolution and human migration patterns (20, 21). Genotypic diversity among KSHV isolates is highest at the left and right ends of the long unique region (LUR) in open reading frames (ORF)-K1 and -K15, respectively (Figure 3). The K1 ORF displays up to 30% amino acid variability, allowing designation of major viral clades A, B, C, D, E, F, and Z. Subtypes B, F, and Z are found almost exclusively in KSHV-infected patients from Africa or of African heritage. Subtype D is found in Pacific Island populations, and subtype C is common in patients from the Middle East and Asia. Both subtypes A and C are seen in North America, and subtype E is present predominantly in ancient populations such as Brazilian Amerindians (Figure 4).

At the opposite end of the viral genome, the K15 ORF can occur in multiple forms that encode for distinct groups of alternatively spliced K15 proteins. These forms, designated as predominant (P) and minor (M) forms (20), apparently arose from rare intertypic recombination events (see reference 22 for a thorough overview of KSHV molecular typing). Internal repeat regions in the latency associated nuclear antigen (LANA)1 gene (ORF73) show strain-to-strain variation in repeat numbers that has also been used for typing (23, 24) and measuring viral monoclonality in KSHV-infected tumors (25).

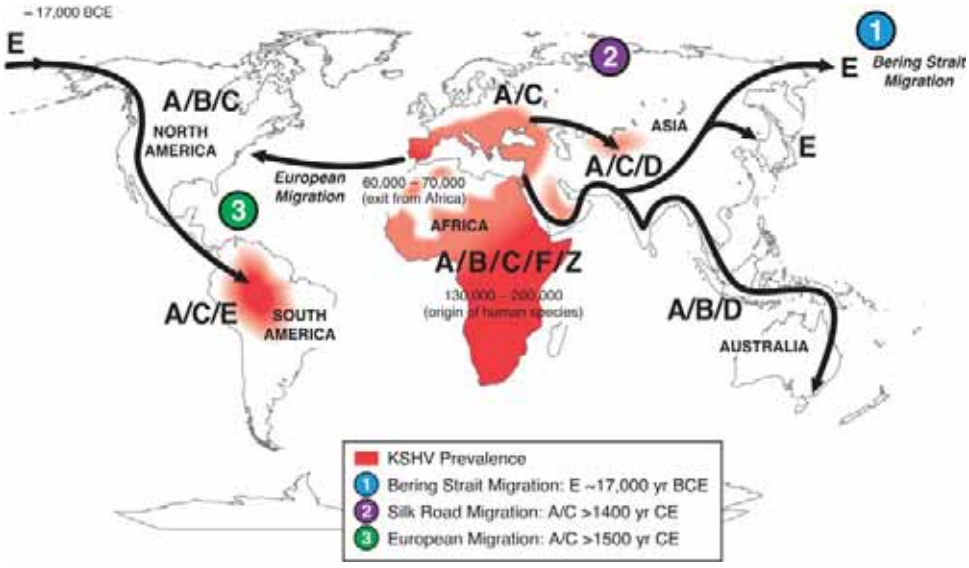

FIGURE 4 The global KSHV seroprevalence, subtype distribution, and migration. KSHV migration might be correlated with the major migrations of modern humans out of Africa over the past 100,000 years.

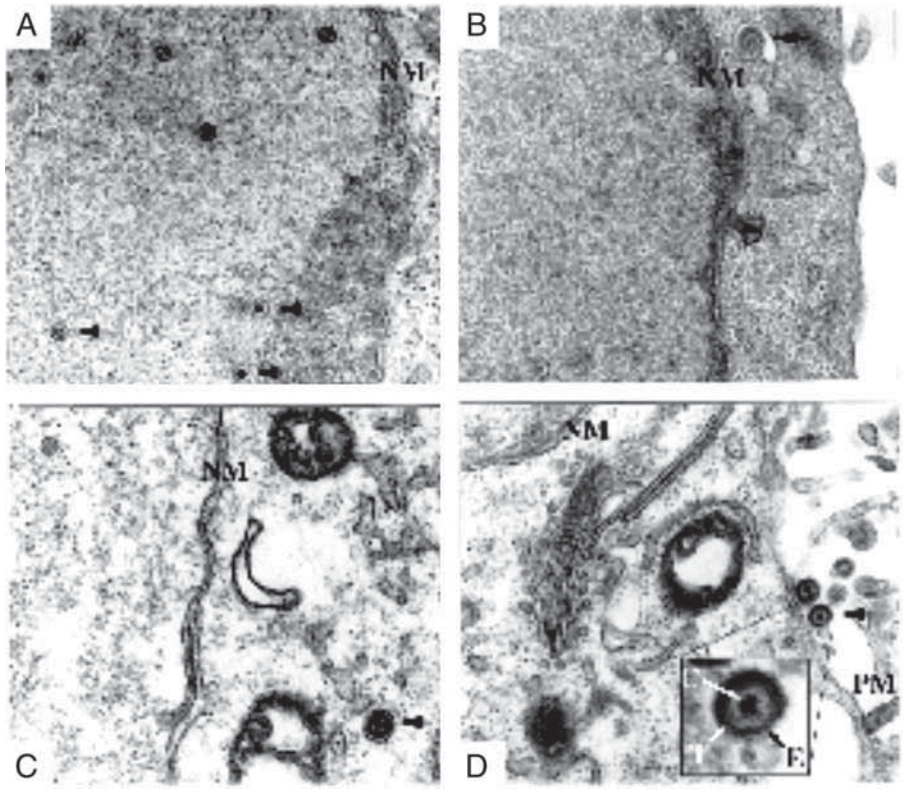

FIGURE 5 Electron photomicrographs of KSHV virion formation and egress in a PEL cell line induced into lytic replication with TPA. A. Naked virus capsids are formed in the nucleus of the cell (NM, nuclear membrane). B. Virions budding through the nuclear membrane and becoming enveloped viruses. C. Transit of the virus through the cytoplasm (arrow). D. egress of the fully enveloped virus from the cell plasma membrane (PM). E. The insert shows a high magnification image of the virus with the capsid (C), tegument (T), and envelop (E). (Photos courtesy of Antonella Tosoni, University of Milan.)

KSHV Structure

Typical herpesvirus virion structures of approximately 100-nm diameter have been detected in the nuclei of KSHV infected B cell lines 24–48 hours after chemical induction (10, 26) (Figure 5). Electron cryomicroscopic reconstruction shows that KSHV has a three-dimensional structure similar to those of other herpesviruses (Figure 6). The capsid shell is composed of 12 pentons, 150 hexons, and 320 triplexes arranged on an icosahedral lattice (27, 28). The primary capsid component is the major capsid protein (ORF25) that forms both the pentons and the hexons (29). Triplex proteins (ORF62 and ORF26 proteins), a small capsomere interacting protein (SCIP, ORF65), and the scaffolding protein (ORF17.5) are other components of the capsid. Similarities in capsid structure and replication mechanisms between herpesviruses and bacteriophage suggest that herpesviruses may have distantly evolved from these prokaryote phage viruses (30).

The space between the virus capsid and the viral envelope is filled with an amorphous protein structure called the tegument, which is organized into three prominent regions: a penton-binding globular region, a helix-bundle stalk region, and a β-sheet-rich triplex-binding region (31). The tegument is composed of specific proteins, such as ORF45 and ORF75 proteins (32, 33), that are microinjected into cells after viral membrane fusion with the host cell and prepare it for infection. Remarkably, the tegument also incorporates a number of viral mRNAs and small noncoding microRNAs

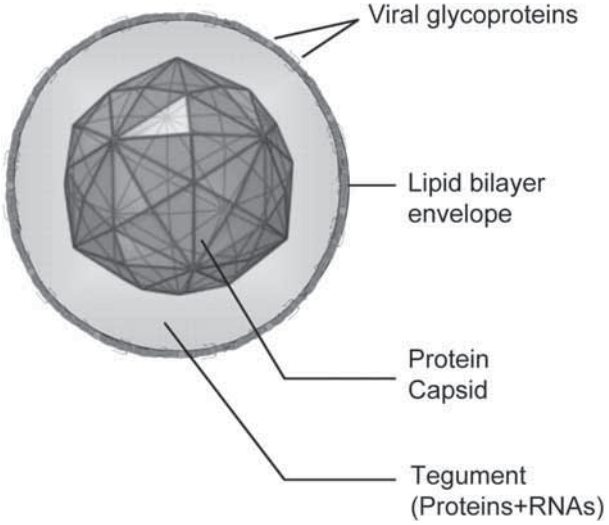

FIGURE 6 Schematic diagram of KSHV enveloped virus structure. The capsid is composed of hexons, pentons, and triplexes arranged in an icosahedral lattice that contains the virus genome. This is surrounded by an amorphous tegument layer composed of viral and cellular proteins and viral RNAs that are microinjected into the cell on infection. The bilayer envelope surrounding the tegument contains viral glycoproteins such as gB that act as receptor and entry proteins.

TABLE 1 KSHV genes with mitogenic or cell cycle regulatory functions

KSHV protein	KSHV gene	Features and functions	References
LANA1	ORF73	Activates multiple mitogenic and oncogenic pathways including β-catenin, c-myc, ERK, hypoxia, surviving, Notch, and BMP-SMAD1-IDs pathways	(191–198)
vCyclin	ORF72	Associates with cdk6 to phosphorylate RB and histone H1; Phosphorylates and downregulates p27; Promotes high density cell proliferation and cellular transformation	(199, 200, 204)
vFLIP	ORF71	Induces lymphoma; Transform rodent fibroblasts; Activate NF-kB; Induce inflammatory cytokines	(215, 222, 223, 289, 290)
vGPCR	ORF74	Induces cellular transformation, angiogenesis, and VEGF in rodent fibroblast; Signals through heterotrimeric G proteins, JNK/Sapk, p38/HOG, and Akt	(291–294)
vIL-6	ORF K2	B-cell proliferation factor; Induces angiogenesis and VEGF in rodent fibroblasts	(101, 211)
vIRF-1	ORF K9	Cellular transformation	(228)
KIST	ORF K1	ITAM-mediated signaling, cellular transformation	(110, 218, 219)
Kaposin	ORF K12	Induces cellular transformation; Activates the p38/MK2 pathway	(295, 296)
K-bZIP/RAP	ORF K8	Inhibits G2/M transition	(48)

(miRNAs) (34–36) that similarly can directly act on their targets once injected into a new cell during the initial stages of infection.

Replication of KSHV

KSHV has both lytic and latent life cycles. During lytic replication, the virus expresses its own DNA polymerase, replicates its own DNA, and generates hundreds to thousands of viral particles per cell over a 24- to 96-hour period, a process thought to invariably lead to cell death. During latent replication, viral DNA exists as a circular nuclear episome that relies on host enzymes for genome replication.

Lytic Replication

Lytic replicating viruses can be seen as massed nuclear inclusions containing electron-dense cores in KSHV-infected cells (10, 26). The virus replicates in the cell nucleus and packages its genome into the viral capsid as a linear molecule that acquires an initial membrane envelope by budding through the inner nuclear membrane. Components of this membrane mature as the virus passes through the outer nuclear membrane and transits through the Golgi apparatus, where the virus incorporates specific viral glycoproteins prior to reaching the plasma membrane. A number of viral proteins including gB, K8.1, ORF33, ORF67, and ORF69 have been identified as participating in this process (37–39). The virus can be released from the cell by direct plasma membrane budding, although release through exocytic vesicles is thought to be more common (40).

The lytic viral genome replicates through a rolling circle mechanism from the circular genome, like the unrolling of a tape, producing a linear strand of concatenated genomes. The KSHV genome concatemer is packaged into the nascent capsid through a portal protein complex (41) and cleaved within the terminal repeat region, resulting in a linear viral genome having variable numbers of terminal repeat sequences at both ends. Lytic viral genome replication is most efficient when cells enter the S phase (40, 42), and KSHV genes expressed during lytic replication act to overcome the G1/S cell cycle checkpoint (35, 43, 44) as well as to disarm host cell innate immune signaling pathways (Tables 1 and 2) (45–47). During late stages of infection, structural component proteins of the capsid are synthesized; the capsid forms on a putative scaffolding that is turned over or incorporated into the capsid as the virion matures.

Rapid viral synthesis after initiation of lytic replication results in many capsids being only partially filled (29), and only a portion of virus produced during lytic replication is fully mature and transmissible. When KSHV lytic replication is initiated, early genes alter cell cycle transit (48) and inhibit cellular mRNA processing and stability to maximize production of viral DNA (49). Simultaneously, nonstructural KSHV gene expression alters the cell to prevent adaptive and innate immune activation. Although antiapoptotic proteins are expressed during lytic virus replication to prolong survival of the infected cell (Table 3) (50–52), damage caused by host protein synthesis shutoff (49), fragmentation of viral DNA (53), and activation of innate immune signaling (54) pathways ultimately lead to host cell death during lytic replication.

Latent Replication

During latent viral replication, the viral genome is present in cells as an extrachromosomal circular episome (plasmid). This may limit activation of DNA damage responses from free viral DNA ends. The viral DNA is replicated in tandem with host DNA by using the host cell machinery so that stable copy numbers of KSHV are maintained in latently infected cells. Latent viral gene expression is highly restricted to viral proteins and miRNAs essential for maintaining the viral episome and preventing premature cell death and senescence (Table 3) (55, 56). The bulk of KS tumor cells infected with KSHV are in a latent state, and less than 5% of tumor cells are actively undergoing lytic replication at any one time.

KSHV Tropism and Primary Infection of Cells

KSHV primarily infects B-cells and endothelial cells but also can infect dendritic cells, macrophages, epithelial cells, fibroblasts, and mesenchymal stem cells in laboratory cell culture. Initially, the virus attaches through electrostatic interactions with heparin sulfate (57) and then initiates

TABLE 2 KSHV genes with immune evasion functions

KSHV protein	KSHV gene	Features and functions	References
vIRF1	ORF K9	Inhibits interferon-related transcription	(228, 229)
vIRF2	ORF K11.1	Inhibits PKR signaling	(235)
LANA1	ORF73	Inhibits IRF3 activation; Prevents MHC I peptide processing; Inhibits CIITA by blocking the IRF-4 binding to pIII and pIV promoters and by binding to RFX complex	(234, 252, 253, 255)
LANA2 (vIRF3)	ORF K10.5	Inhibits IRF7 activation; Antagonizes PKR signaling; Inhibits the PML-mediated transcriptional repression; Inhibits expression of interferon-γ and CIITA	(236, 243, 254)
RTA	ORF50	Targets IRF7 for degradation	(231)
ORF45	ORF45	Blocks IRF7 activation to inhibit type I interferon induction	(233)
vIL-6	ORF K2	Abrogates signaling from the interferon-β receptor; Polarizes anti-KSHV immune signaling towards Th2 response	(101, 260, 261)
vMIP-I	ORF K6	Chemotaxis; Regulates Th2 response and angiogenesis	(297)
vMIP-II	ORF K4	Chemotaxis; Polarizes anti-KSHV immune signaling towards Th2 response	(260, 261)
vMIP-III	ORF K4.1	Chemotaxis; Polarizes anti-KSHV immune signaling towards Th2 response; Acts as a CCR4 agonist and stimulates angiogenesis	(260, 261)
RIF	ORF10	Blocks Jak1/Tyk2-STAT2 interferon signaling	(238)
ORF63	ORF63	Inhibits NLRP1-dependent innate immune responses	(241)
ORF64	ORF64	Reduces RIG-I ubiquitination to inhibit its activation and suppresses RIG-I-mediated interferon signaling	(239)
MIR1	ORF K3	Functions as an E3 ligase; Downregulates surface expression of MHC I; Inhibits cell surface presentation of T cell costimulatory molecule B7.2, NKT cell CD1 signaling, and interferon-γ receptor	(237,248–250 257, 258)
MIR2	ORF K5	Functions as an E3 ligase; Downregulates surface expression of MHC I, NKG2D ligands MICA and MICB, and NKp80 ligand AICL; Inhibits cell surface presentation of T-cell costimulatory molecule B7.2, NKT cell CD1 signaling and interferon-γ receptor	(237,248–250257–259)
ORF75	ORF75	Antagonizes ND10-mediated intrinsic immunity	(242)
KIST	ORF K1	ITAM-mediated signaling; Mimics activated B-cell receptor (BCR)	(218, 219, 247)
vCBP	ORF4	Complements inhibitory protein; Mimics BCR signaling; Promotes survival of infected B cells	(220)
miR-K1	miR-K1	Downregulates IκBα; Activates NF-κB pathway	(298)
miR-K11	miR-K11	Decreases IKKε-mediated IRF3/IRF7 phosphorylation; Inhibits the activation of IKKε-dependent ISGs	(232)

membrane binding (through interactions with integrins α1β3, αvβ3 and EphrinA2) (58–60) and membrane fusion (through xCT interactions) (61). The presence of these receptors on the cell surface largely determines whether a host cell is susceptible to KSHV infection.

KSHV-receptor interactions activate a number of cellular pathways, including MEK/ERK, JNK, p38, and FAK pathways, that are needed for KSHV entry and transport to the nucleus (62–65). KSHV enters cells by endocytosis (66–68) and then undergoes activated intracellular transport by the cytoskeletal machinery to perinuclear regions, where it delivers the viral genome into nuclei (67, 69, 70).

The extent of cell-specific activation of signaling pathways during infection determines the expression of viral genes and the scale of viral lytic versus latent replication. Robust induction of the MEK/ERK, JNK, p38, and MSK pathways promotes lytic replication by activating the AP-1 and CREB1 transcriptional complexes, while the NF-κB pathway suppresses KSHV lytic replication and promotes viral latency (63, 65, 71, 72). In human foreskin fibroblasts and dermal microvascular endothelial cells, KSHV has a short and limited scale of lytic gene expression and quickly enters latency upon infection (73). In human umbilical vein endothelial cells, KSHV has a robust lytic replication program producing high titers of infectious virions before entering into latency (35, 74).

Latent and Lytic Gene Expression

Initiation of lytic replication begins with a highly choreographed cascade of gene expression that determines the sequence of events leading to linear genome replication, virion synthesis, genome packaging, and egress of the virus from the infected cell (75, 76). Lytic replication begins with immediate-early expression of the replication and transcription activator protein, RTA, encoded by ORF50 (75, 77). RTA in combination with the transcription factor RBP-Jκ activates its own promoter (78), resulting in a positive feedback loop to generate sufficient RTA to maintain the remaining steps in lytic or productive virus replication. PKC, ERK, JNK, p38, MSKs, H-Ras, hypoxia, and ER stress promote KSHV lytic replication by inducing RTA expression through activation of transcriptional factors including c-Fos, c-Jun, CREB1, Ets-1, HIFs, and XBP-1 (Figure 7) (63, 65, 72, 79–84).

Viral lytic gene expression is associated with increased histone activation marks such as histone H3 trimethyl Lys4

TABLE 3 KSHV genes with anti-apoptotic and anti-autophagic functions

KSHV protein	KSHV gene	Features and functions	References
vFLIP	ORF K13	Activates canonical and noncanonical NF-kB pathways; Promotes cell survival; Induces spindle morphology; Inhibits autophagy by preventing Atg3 from binding and processing LC3	(205–207, 221–223 289, 290)
vBCL-2	ORF16	Inhibits Bax-mediated apoptosis; Suppresses cellular autophagy pathway by interacting with Beclin1 complex	(50, 215)
vIL-6	ORF K2	Act as a B-cell proliferation factor; Prevents apoptosis in IL-6-dependent plasmacytoma cells; Induces angiogenesis and VEGF in rodent fibroblasts	(101, 211)
LANA1	ORF73	Promotes cell survival by inhibiting p53 and p73 pathways and TGF-β signaling	(109Santag, 2013 #611, 299)
LANA2	ORF K10.5	Inhibits p53 mediated transcription	(99)
vIAP	ORF K7	Inhibits apoptosis by binding to Bcl2 via BH2 domain and blocking caspase-3 activity via BIR motif; Interacts with rubicon to inhibit autophagosome maturation by blocking Vps34 enzymatic activity	(51, 216)
vIRF-1	ORF K9	Inhibits p53; Induces Bim nuclear translocation to inhibit apoptosis	(214, 300)
vIRF-4	ORF K9	Inhibits p53	(301)
K-bZIP	ORF K8	Inhibits p53	(213)
RTA	ORF50	Inhibits p53; Induces autophagy during viral lytic replication	(302, 303)
miR-K1	miR-K1	Activates NF-κB pathway by downregulating IκBα	(298)
miR-K10	miR-K10	Inhibits TGF-β signaling and TGF-β-induced apoptosis	(115)
miR-K11	miR-K11	Inhibits TGF-β signaling and TGF-β-induced apoptosis	(304)

(H3K4me3) and decreased repressive histone marks such as H3 trimethyl Lys27 (H3K27me3), resulting in chromatin unwinding (85, 86). Similarly, phorbol esters and histone deacetylase inhibitors that cause chromatin modifications can induce KSHV reactivation (87–90). The most abundant KSHV transcript during lytic replication, the long noncoding PAN RNA (polyadenylated nuclear RNA, ORFK7), is involved in epigenetic regulation of viral and cellular gene expression by interacting with demethylases UTX and JMJD3 and PRC2 complexes (91, 92). PAN RNA is required for viral reactivation in part by binding to LANA1 and sequestering LANA1 away from the KSHV genome to counter its repressive effects on lytic gene expression (92, 93). Cellular molecules identified as negative regulators of lytic replication promote viral latency and include KAP1, HEY1, SIRT1, and several miRNAs (88, 94–97).

Latent KSHV gene expression, in contrast, is constitutive, and not dependent on the replication status of the virus, although it may be regulated by cell cycle stage or tissue type (98–100). Individual genes are often referred to as "latent" or "lytic" genes, although in reality their expression patterns are controlled by a number of factors beyond the KSHV replication cycle. Some viral genes encoding proteins, such as vIL-6, that are induced during lytic replication are also induced entirely independently of lytic replication (101). Notch signaling has been demonstrated to induce a wide variety of KSHV nonstructural genes without lytic viral replication (so-called class II genes) (102, 103). KSHV has many different gene expression profiles that depend on virus replication status, cell and tissue type, immune signaling and other factors—a complexity that is obscured by dichotomizing KSHV gene expression into only latent and lytic forms.

Organization and Features of the KSHV Genome

The KSHV genome was first sequenced from the virus-infected lymphoma cell line BC-1 (11). Because of the extensive collinear homology between KSHV and the first sequenced rhadinovirus, herpesvirus saimiri, KSHV genes (or ORFs) are named according to their herpesvirus saimiri positional homologs (e.g., ORFs 4–75) if obvious gene similarity is present (Figure 3). Those genes unique to KSHV are given a K prefix starting from the left end of the genome (e.g., ORFs K1-K15) (11). KSHV is unique among the human herpesviruses in the extent of its extensive molecular piracy of genes from the host genome. These stolen genes include not only DNA replication genes found in other herpesviruses but also regulatory, cytokine, receptor, and immune signaling genes that contribute to KSHV-related pathogenesis.

The long unique region (LUR) is approximately 145 kilobases in size and possesses close to 90 viral genes thus far identified (Figure 3). Structural (e.g., capsid) and lytic replication genes common to other herpesviruses are present in the KSHV genome and tend to be grouped together. These KSHV genes generally share the highest degree of homology with other herpesvirus genes. Conservation of structural proteins leads to cross-reactivity with virion structural antigens of other herpesviruses in serologic assays, although this problem can in part be overcome by identifying unique antigens through peptide mapping (104–107).

DNA polymerase (ORF9), thymidine kinase (ORF21), and phosphotransferase (ORF36) (108) are conserved with other herpesviruses and are targets for antiherpesvirus drugs. KSHV has other DNA synthesis enzymes that are not found in most other herpesviruses, including a dihydrofolate reductase (ORF2), a ribonucleotide reductase (ORF66), and a thymidylate synthase (ORF70). The cellular counterparts to these genes are regulated by the retinoblastoma cell cycle checkpoint protein. KSHV probably uses these viral enzymes to overcome the G1/S checkpoint during lytic replication or by inducing an artificial S phase, which can allow viral DNA replication. These genes are also potential drug targets against lytic virus production.

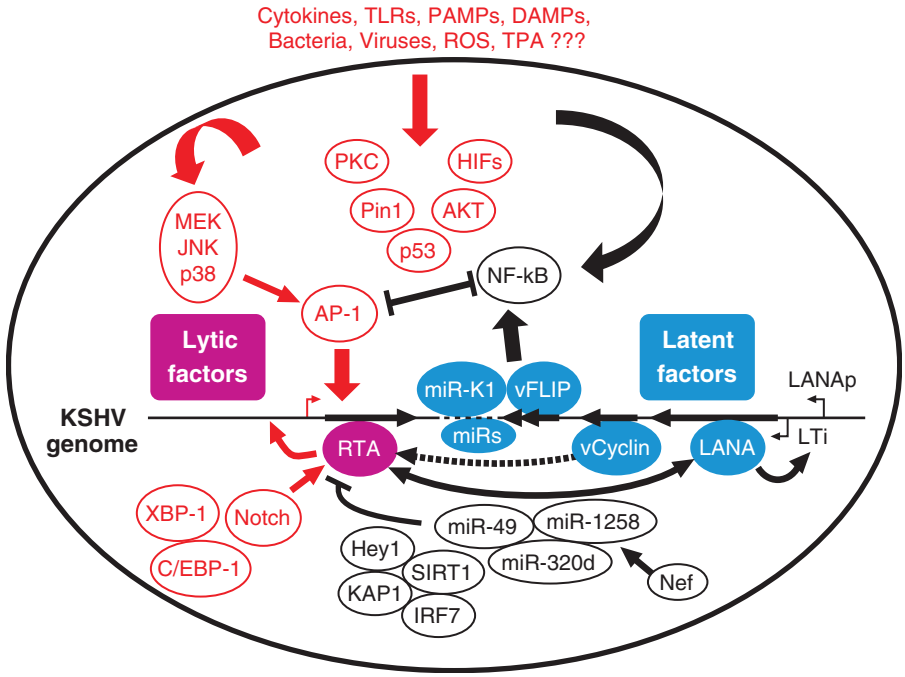

FIGURE 7 Schematic illustration of mechanisms of KSHV latency and reactivation. Expression of KSHV latent products including LANA, vCyclin, vFLIP, and miRNAs enhance/maintain latency by inhibiting KSHV lytic replication, promoting cell survival, and facilitating the viral episome replication and segregation. Several cellular factors such as NF-κB, Hey1, SIRT1, KAP1, and IRF7 as well as cellular miRNAs inducing miR-1258 and miR-320d repress KSHV lytic replication. In contrast, several physiological factors including hypoxia, HIV infection, inflammatory cytokines, oxidative stress, and ROS can induce RTA expression by activating specific cellular pathways and transcriptional factors including MEK/ERK, JNK, p38, AP-1, MSKs, Ets-1, Pin1, HIF1/2, PKC, and Notch. RTA interacts with several host proteins such as XBP-1, Notch, and C/EBPα, as well as viral proteins such as MTA and kb-ZIP to induce the expression of viral lytic genes and activation of the entire viral lytic transcriptional program.

While genes required for lytic replication are largely conserved with other herpesviruses, those genes expressed during latency are unique to KSHV. KSHV, for example, does not possess direct sequence homologs to any of the EBV latency genes, such as the Epstein-Barr nuclear antigens (EBNAs) or latent membrane proteins (LMPs). Despite these genetic differences, KSHV and EBV tend to target the same regulatory cellular circuits. In general, KSHV has pirated, modified, and targeted cellular genes for use during its replication and survival, while EBV tends to regulate these same pathways using complex viral transcriptional activating proteins. As an example, KSHV encodes an IL-6 homolog whereas EBV LMP1 induces expression of cellular IL-6; many other parallel examples exist. Conserved and nonconserved genes are segregated into blocks of genes, which tend to be regulated together.

The KSHV pirated genes express proteins that function similarly to host proteins but have modified regulatory functions. Known homologs to cellular genes include a complement binding protein (KCP), vIL-6, viral macrophage inflammatory proteins (vMIPs or viral CC-chemokine ligands, vCCLs), vBcl-2, viral interferon regulatory factors (vIRF1, vIRF2, LANA2, and vIRF4), a viral FLICE-inhibitory protein (vFLIP), a viral cyclin (vCyclin), a viral adhesion molecule (vOX2 or vAdh), and a viral G-protein-coupled receptor (vGCR, ORF74). Additional KSHV proteins, such as LANA1 and KIST (KSHV immunosignaling transducer or ORF K1 protein), do not have known cellular homologs but have roles similar to those of oncoproteins found in other viruses (109, 110).

KSHV encodes a large number miRNAs that are expressed during latency (111). A number of KSHV miRNAs share seed sequences with cellular miRNAs. KSHV miR-K11 mimics miR-155 (112, 113), miR-K10a/b and variants mimic miR-142-3p variants (114, 115), and miR-K3 (variant miR-K3+1) mimics miR-23a/b/c (116). KSHV miRNAs mediate viral latency by suppressing lytic replication, and promote cell growth and survival (56).

Recent mapping of KSHV transcripts and 3' UTRs by high throughput sequencing (117–120) have revealed a far more complex KSHV transcriptome than previously described (103): over one third of the genes may be bicistronic and polycistronic. The extended 3' UTRs of these transcripts are presumably regulated by a variety of mechanisms including targeting by miRNAs, providing additional layers of unexpected complexity in controlling viral gene expression (121).

EPIDEMIOLOGY

KSHV-related diseases are a major unmet public health problem. Although the incidence of AIDS-associated KS declined 70% to 90% after the introduction of effective antiretroviral therapy, these drugs do not inhibit KSHV infection and a second emergence of KS among persons with low HIV loads and high CD4+ cell counts has been reported (122, 123). Clinical manifestations such as immune reconstitution inflammation syndrome (IRIS)-KS in the era of cART pose additional new challenges (1, 124). Finally, although KSHV is poorly transmissible through transfusion,

TABLE 4 Patterns of KSHV infection and KS prior to the AIDS epidemic

KS incidence	Regions	Population KSHV prevalence	Transmission	Risk groups
Low	North America, North Europe, East Asia	0–5%	Sexual, iatrogenic	Homosexual men, STD attendees, transplant recipients
Intermediate	Mediterranean, Middle Eastern countries, Caribbean	5–20%	Sexual, iatrogenic, nonsexual	Homosexual men, STD clinic attendees, transplant recipients, older adults
High	Africa	>50%	Nonsexual, sexual	Children, older adults, lower socioeconomic status

this route of transmission can occur and may cause silent infection and disease (125, 126).

Distribution and Geography

Unlike other human herpesviruses, KSHV is an uncommon infection (~2% to 3%) in North American and northern European populations (Table 4) (127). Rates of infection are higher among Mediterranean, Eastern European, and North-Western Chinese populations, and highest in African populations, particularly Central-Eastern African populations (9, 128–131). This correlates generally with the worldwide distribution of KS disease prior to the emergence of the AIDS epidemic (Figure 4) (129). There are population-wide correlations between KSHV infection and KS disease rates, as seen by comparing provincial Italian KSHV infection rates in blood donors with historic pre-AIDS rates of KS disease with increasing rates of infection and disease occurring in a north-to-south gradient along the Italian peninsula (128, 132). Not all studies, however, find a close geographic correlation between infection rate and disease. Despite overall low infection rates in the Americas, very high KSHV infection rates are present among isolated South American indigenous populations (133–135). Viral genotypes isolated from these Amerindian populations most closely match KSHV strains isolated in Asia, suggesting that the virus first migrated with humans to the Americas across the Bering Strait thousands of years ago (Figure 4) (136).

Transmission

Sexual Transmission

In American and European populations, the groups at highest risk for KSHV infection are men who have sex with men (MSM), in whom infection rates range from 30% to 60% (Table 4) (9, 130, 131). While compelling evidence indicates that KSHV is sexually transmitted (Figure 8), the specific behaviors resulting in KSHV transmission are disputed. Heterosexual transmission appears to be uncommon in these areas.

KSHV is not appreciably shed into semen and direct transmission through unprotected anal intercourse is unlikely. KSHV, like most other herpesviruses, is asymptomatically shed from the oropharynx (137). A significant risk factor for virus transmission among homosexual men has been found to be deep kissing, although oral-penile contact and oral-anal contact are also significant risk factors for infection or seroconversion (138, 139). Since heterosexual couples also engage in kissing and oral-penile contact, the reasons MSM couples are much more susceptible to infection remain unclear. Use of saliva as a sexual lubricant for anal intercourse may also contribute to high infection rates.

Nonsexual Transmission

Explanations for the extremely high endemic rates of KSHV and KS in sub-Saharan Africa are also lacking. KSHV infection is common among presexually active African children. Areas of relatively high endemicity in Europe, including Italy, also show evidence for horizontal nonsexual transmission among children and possibly among adults (140). Congenital and perinatal mother-to-child transmission is rare, but transmission from mothers to older infants and children may be more common (141, 142), presumably related to oropharyngeal shedding and saliva contact (143, 144).

Initial investigations of KSHV among AIDS patients found that persons infected with HIV through blood products had low rates of KS (5). This led to the assumption that the agent causing KS is not transmitted by transfusion. However, case-control studies demonstrate that the virus is detectable in approximately one-half of peripheral blood mononuclear cell (PBMC) samples from AIDS-KS patients using standard PCR techniques (145), and the rate of virus detection may be dependent on the level of host immunosuppression (146).

Direct demonstration of bloodborne transmission has been found in both U.S. and African studies (2, 125, 126). Transfusion transmission is less efficient than for other viruses (~4% per infected transfusion) (126) and may be markedly reduced by blood processing. Despite the low

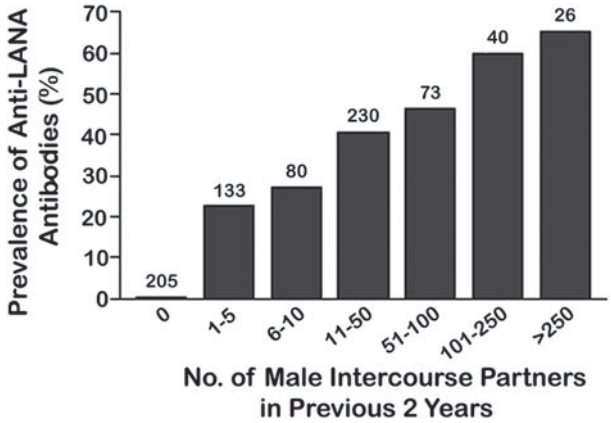

FIGURE 8 KSHV seroprevalence increases linearly with numbers of recent sex partners in this population-based sampling of gay and bisexual men (sera collected in 1984) from San Francisco. This and related risk-factor data suggests that KSHV is sexually transmitted although the precise mechanism for transmission remains unclear. (From Martin et al., (319) with permission.)

efficiency of bloodborne transmission, large numbers of transmission events might take place in the United States alone each year.

Transplantation

KSHV transmission during transplantation presents the most immediate public health concern that is amenable to simple interventions. Initial high rates of transplantation-related KS have been reported (147, 148) but these rates have precipitously declined with lowered immune suppression regimes (149). European studies demonstrate KS resulting from transplantation is principally due to reactivation of KSHV infection. Approximately one third of transplant KS patients are infected from the donated organ (150). The rate of disease among KSHV-positive transplant patients can be strikingly high, between 25% and 68% (151, 152). Intriguingly, some KS tumors are derived from the donor rather than the recipient, suggesting that transmission of tumor cells rather than infectious virus contributes to transplantation KS (151, 152). Transmission can occur after transplantation of any organ including heart, bone, as well as liver and kidney, and all KSHV-related diseases including KS, PEL, MCD, and bone marrow failure, have been reported in the transplant setting. Mortality and graft loss is high among transplant KS patients (153), strongly arguing for primary screening.

African KS Epidemic

Prior to the AIDS epidemic, KS was the third most commonly reported tumor in some areas of Africa (Figure 9). With the onset of the AIDS epidemic, rates of KS have dramatically risen and it is now the most commonly reported cancer in many sub-Saharan African countries. In Zimbabwe, for example, KS accounts for 31% of cancers (154) and in Uganda it is the most common tumor in males and the second most common in females (behind cervical cancer) (155). Unlike the North America and Europe, men and women have similar KSHV infection rates in African countries (156). KS tumors, however, are more common in men, leading to speculation for a role of sex hormones in disease expression.

KS is virtually nonexistent among children from developed countries, but it is one of the most common cancers among African children (157). Pediatric KS is a fulminant disease that spreads via the lymphatic system, generally resulting in death within a year (157). As previously indicated, horizontal transmission appears to be the predominant means of infection (142).

CLINICAL MANIFESTATIONS AND PATHOGENESIS

Primary Infection in Humans

Primary KSHV infection is generally asymptomatic; rare cases of seroconversion-associated atypical lymphocytosis with mononucleosis-like symptoms (158) and rashes with or without fever have been reported in immunocompetent children from regions of high KSHV seroprevalence (159, 160). In immune-suppressed individuals, KS can develop within weeks or months of primary infection. For transplant patients infected by organ donation, KSHV-related diseases can erupt within 2 to 6 months of transplantation, indicating that the incubation period for KS is primarily dependent on the state of immune surveillance rather than length of time of infection. Among Dutch and American homosexual cohorts with both HIV and KSHV infection, those persons infected with KSHV first and HIV second tend to have a lower rate of developing KS (161, 162), suggesting that HIV-induced immune dysfunction impairs immunologic surveillance and control for KSHV.

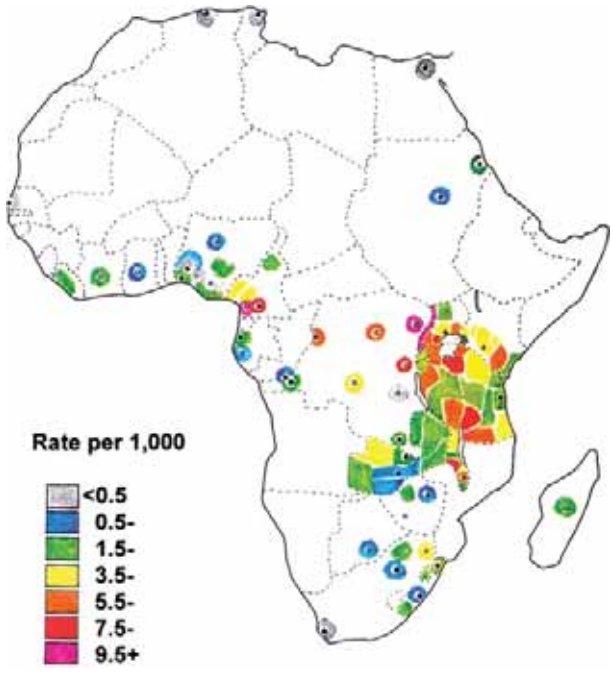

FIGURE 9 Map of KS prevalence throughout Africa prior to the AIDS epidemic. This map was constructed from surveys performed by Denis Burkitt who first described Burkitt's lymphoma. It is evident that KS was hyperendemic throughout this continent. With the onset of the AIDS epidemic, a second epidemic of KSHV-related cancer has occurred and KS is the most commonly reported cancer in most sub-Saharan African countries.

Kaposi's Sarcoma

KS is a complex tumor characterized by proliferation of spindle-shaped, KSHV-positive endothelial cells forming abnormal slit-like vascular channels. KS lesions frequently contain inflammatory infiltrates comprised of lymphocytes and macrophages suggesting an immune response against tumor-specific antigens. Neoangiogenic vessels can also be seen in KS tumors but these are negative for KSHV and are distinct from the disorganized tumor endothelial cells (163).

KS often first appears on the skin (Figure 10), particularly of the extremities, face, and genitalia but can disseminate to mucosal surfaces, lymphoid tissues, and viscera, especially in immunocompromised patients. KS skin lesions initially appear as bruised or discolored macules that progress to nodules and plaques, often with ulceration in late stages of disease. Advanced disease may be associated with edema and spread to surrounding lymph nodes. Gastrointestinal dissemination is frequently asymptomatic and can be difficult to diagnose because of submucosal infiltration by the tumor. Pulmonary involvement has a markedly worse prognosis than isolated skin or mucocutaneous disease and also may be difficult to distinguish radiologically from other opportunistic infections.

At initial presentation, KS lesions can be confused with other conditions or even simple bruises "that don't go away." The Koebner phenomenon, where KS tumors appear at the site of an old scar, is not uncommon. The differential diagnosis, particular among HIV/AIDS patients, should include

bacillary angiomatosis (due to *Bartonella*) and even skin manifestations of *Mycobacterium haemophilum*. KS tumors can occur in combination with MCD in lymph nodes or with PEL in visceral cavities, resulting in a mixed tumor cell population and complex clinical presentation.

All forms of KS (classical, endemic, epidemic, and iatrogenic) are KSHV positive, histologically indistinguishable, and differ only in clinical or epidemiologic characteristics. Classical KS is generally an indolent tumor occurring in elderly patients, particularly men of Mediterranean, Eastern European, or Middle Eastern ethnicity. No specific risk factors for classical KS have been identified although country or region of birth (139, 164) and receipt of blood transfusions (165) may be associated with elevated disease risk. HIV-seronegative homosexual men also appear to be at increased risk for classical KS (166).

Endemic or African KS is similar to classical KS in having a male predominance but can be a progressive and rapidly fatal disease, especially in children. While the geographic pattern of occurrence in Africa overlaps with EBV-associated Burkitt's lymphoma, areas with high rates of KS and Burkitt's lymphoma are not identical (167). Endemic KS is not associated with specific immunodeficiency although some patients may have diminished responses to skin test antigens (168).

AIDS-KS or epidemic KS is clinically aggressive, and visceral involvement is common. Male homosexual and bisexual AIDS patients are approximately 20 times more likely to present with KS at AIDS diagnosis than hemophilic AIDS patients (5), and cohort studies demonstrate that up to one half of homosexual/bisexual men with AIDS develop KS over the course of their lifetimes. The reported proportion of U.S. AIDS patients with KS as an initial AIDS-defining condition has declined since the beginning of the AIDS epidemic, perhaps due to changes in sexual practices (5) and the use of potent antiretroviral therapy. However, there is an increased incidence of KS-IRIS, which often has high mortality rates, particularly in sub-Saharan Africa (1).

Primary Effusion Lymphomas (PEL)

Primary effusion lymphomas (PEL) are a rare type of non-Hodgkin B cell lymphomas first described in AIDS patients as body cavity-based lymphomas (BCBL) (169). PEL are infected by KSHV at high copy number (50 to 150 viral genome copies/cell). The KSHV populations infecting a particular PEL are monoclonal by viral terminal repeat

FIGURE 10 A. Typical appearance of KS lesions on arm and chest for an AIDS patient. B. KS frequently involves mucosal surfaces, in this case sublingual palette. C. Disseminated skin KS occurring in a dermatomal distribution on the back of an AIDS patient. D. An AIDS patient's leg showing post-radiation hyperpigmentation, ulceration, and nodular KS lesions that have recurred within the radiated area. (Photos courtesy of Bruce Dezube, Beth Israel Deaconess, Boston, MA, and Susan E. Krown, Memorial Sloan-Kettering Cancer Center, NY, NY.)

analysis, indicating that each lymphoma arises from a single KSHV infected cell. Although PELs are most frequently observed in the setting of AIDS and coinfected with EBV, PELs solely infected with KSHV have been described for both HIV-positive and HIV-negative patients (170). PELs characteristically present as intracavitary pulmonary, abdominal, or cardiac effusions with tumor cells growing in suspension. Usually there is no direct local tumor destruction or invasion into serous membranes although spread to lymph nodes and lymphatics occurs, and circulating lymphoma cells can be isolated from the peripheral blood (9, 171). Extracavitary PEL have been reported (172).

PEL are composed of postgerminal center, preterminally differentiated malignant B-cells. Consistent with their plasmablastic stage of differentiation, the PEL tumor cell phenotype is inclusive of CD45 and CD138/syndecan-1 but exclusive of CD19, CD20, and CD79a/b antigens (173). Although PEL do not usually express surface B-cell markers, they have clonal immunoglobulin chain rearrangements and display lymphocyte activation markers including CD30, CD38, CD71, and epithelial membrane antigen (7, 169, 174). PEL tumor cells have morphologic features that span those of large cell immunoblastic lymphomas and anaplastic large cell lymphomas with abundant atypical mitosis, marked nuclear/cytoplasmic pleomorphism, and prominent nucleoli. These lymphomas consistently lack genetic alterations that have been associated with other lymphomas, including activation of the proto-oncogenes c-Myc, Bcl-2, and Ras as well as mutations of p53 (169, 175). However, recurrent chromosomal gains have been detected in six chromosomes (176). A search for driver mutations that complement KSHV in PEL tumorigenesis has revealed strong evidence that mutations to X-chromosone-linked interleukin 1 receptor-associated kinase (IRAK1) are likely to be critical to PEL etiology (320).

The majority of PEL are described in patients with compromised immune status, especially those with AIDS. In these individuals, PEL respond poorly to therapy and are highly aggressive, often rapidly fatal with a median survival of 2 to 3 months after initial diagnosis (177). Symptoms are frequently due to the mass effect of the rapidly expanding malignant effusion on adjacent vital organs, and drainage procedures can provide short-term symptomatic and functional relief. In contrast, PEL often have a much less aggressive course in HIV-seronegative patients, and they tend to occur in elderly persons.

Multicentric Castleman's Disease

Castleman's disease is a rare non-neoplastic, lymphoproliferative disorder related to excess IL-6-like activity. It encompasses a spectrum of clinicopathologic entities that range from circumscribed involvement of localized lymph nodes, referred to as unicentric Castleman's disease (UCD), to generalized lymphadenopathy, also called multicentric Castleman's disease (MCD) (178). Castleman's disease patients can be infected by KSHV with or without HIV coinfection or may be found in patients with no evidence of infection with either virus. Histologically, Castleman's disease is divided into two subtypes: hyaline-vascular and plasma-cell variants, but mixed forms of the two can be juxtaposed in a single lymph node. The majority of the hyaline-vascular variant occurs in a clinical setting of UCD and presents as solitary lymph node hyperplasia typically in the mediastinum or retroperitoneum. Other than isolated nodal enlargement, patients have few other symptoms and surgical excision of involved lymph nodes is curative. In contrast, the plasma cell histologic variant is most frequently correlated with multicentric or generalized involvement, and is challenging to treat. Clinically, MCD patients present with a variety of proinflammatory systemic symptoms including fever, night sweats, and fatigue. Physical examination is often notable for cachexia and hepatosplenomegaly in addition to generalized lymphadenopathy. Laboratory findings include anemia, hypergammaglobulinemia, hypoalbuminemia, elevated inflammatory markers, and high circulating levels of IL-6 and IL-10. Intercurrent diseases frequently diagnosed with MCD or during follow up include Kaposi's sarcoma, lymphomas, and hemophagocytic lymphohistiocytosis (HLH) (179, 180).

KSHV infection is present in essentially all cases (99.7%) of MCD occurring in HIV-infected individuals; and in approximately a quarter to half of MCD in HIV-seronegative patients (181). In MCD, KSHV infected cells have a distinctive distribution. Immunohistochemical localization studies show that KSHV-infected cells comprise a minority population of B-cells in the mantle zones, which surround germinal centers (182–184). These infected cells have a plasmacytoid immunoblastic or plasmablastic morphology and express abundant KSHV vIL-6 (182) supporting the notion that Castleman's disease is a syndrome of multiple etiologies involving aberrant IL-6 activity.

Other Disorders Associated with KSHV

KSHV is identifiable in several rare lymphoproliferative disorders in addition to PEL, which can be broadly grouped as PEL-like solid lymphomas, plasmablastic proliferations arising in MCD, and germinotropic lymphoproliferative disorder. Descriptive names given these entities include KSHV-associated large B-cell lymphoma (KSHV-LBL), intravascular large B-cell lymphoma, and HHV8+ plasmablastic microlymphoma/lymphoma (172, 185, 186). The literature suggests a significant degree of overlap in these entities with PELs and MCD in terms of phenotypic markers, EBV status, and HIV status. Non-neoplastic diseases including bone marrow failure and some forms of autoimmune hemolytic anemia in the setting of Castleman's disease have been attributed to KSHV infection (19, 182).

Pathogenesis

The specific mechanisms by which KSHV induces tumor formation remain incompletely defined and vary in each of its associated diseases. KSHV is a monoclonal infection in PELs, which are clearly composed of fully transformed B cells (11, 171, 187). KS lesions, in contrast, can be oligoclonal or polyclonal but probably evolve into monoclonal lesions with disease progression (11, 188). The bulk of cells in KSHV Castleman's disease MCD lesions, in further contrast, consist of hyperplastic, uninfected, untransformed cells. Only a fraction of cells, localized to the marginal zone of germinal centers, are infected with KSHV. Interestingly, while these infected cells are not monoclonal, they tend to show monotypic lambda light chain expression (189). Among the three main KSHV-associated human diseases, the mechanistic underpinning of KSHV MCD is most accessible, can be attributable to KSHV vIL-6 expression, and even has a naturally occurring human disease counterpart, KSHV negative MCD. KSHV MCD is the first recognized disorder that is likely to be caused by a virus-encoded cytokine.

Cell Transformation and Proliferation

KSHV is primarily in a latent, unencapsidated, and nontransmissible form in KS lesions and PELs. While a small

percentage of tumor cells undergo productive virus replication at any one time, the bulk of tumor cells are noninfectious. Therefore, genes expressed during latency are likely to play important roles in KSHV-induced malignancies. Several of these genes have oncogenic functions (Tables 1 and 3). For example, LANA1 is functionally similar to SV40 T antigen in that it targets and inhibits both p53, which controls apoptotic signaling (109), and retinoblastoma protein (pRB1), which is responsible for cell cycle regulation (190). LANA1 has been reported to activate a myriad of mitogenic and oncogenic pathways including β-catenin (191), c-myc (192, 193), ERK (194), hypoxia (195), survivin (196), Notch (197), and BMP-SMAD1-IDs (198) pathways. vCyclin affects the cell cycle in inducing pRB1 phosphorylation by partnering with cyclin-dependent kinases (199, 200). The functions of vCyclin appear to be very similar to those of the cellular class of D cyclins, although vCyclin can also induce phosphorylation of H1 (201, 202). This suggests that vCyclin may be active at stages of the cell cycle other than the G1 checkpoint controlled by pRB1. KSHV vCyclin has been shown to counter the senescence/G1 arrest response triggered by NF-kB hyperactivation (203) and to promote KSHV-induced cellular transformation by overriding contact inhibition (204). Activation of the NF-κB pathway and inhibition of the autophagy pathway by vFLIP are essential for the survival of latent cells (205–207). Several KSHV miRNAs provide oncogenic and prosurvival signals (Table 5). Deletion of a cluster of 10 pre-miRNAs triggers cell cycle arrest and apoptosis in KSHV-transformed cells (208). Thus, these viral miRNAs are required for maintaining the homeostasis of KSHV-transformed cells.

Several viral proteins traditionally associated with lytic replication also activate oncogenic pathways and promote cell survival. vGCR and vIL-6 have mitogenic activity that promote cell cycle entry and may lay the cell cycle groundwork for genome replication (Table 1). Surprisingly, some KSHV proteins, such as K-bZip appear to arrest the cell in late G1 by interacting with the cyclin-dependent kinase CDK2 (48) and by activation of the cyclin-dependent kinase inhibitor p21 (209). This is counterintuitive to the goal of generating an S-phase state for the infected cell and raises the interesting possibility that aberrant arrest may benefit viral DNA over cellular DNA replication.

Many of the functions of the proteins encoded by the viral homologs of cellular genes affect apoptotic signaling. KSHV viral inhibitors of apoptosis including K-bZip, vIRF-1, RTA, vBcl-2, vIL-6, and vIAP are expressed at high levels after KSHV enters lytic replication, and which effectively

TABLE 5 Cellular and viral targets of KSHV miRNAs

Target	Viral or cellular	miRNA	Putative function	Ref
BACH1	Cellular	miR-K11	Countering of oxidative stress	(112, 113)
BCLAF1	Cellular	miR-K5, -K9, -K10a/b	Inhibition of caspase activity	(305)
Bcr	Cellular	miR-K6-5	Promotion of angiogenesis	(306)
CASP3	Cellular	miR-K1, -K3, -K4-3p	Inhibition of apoptosis	(307)
CDKN1A/p21	Cellular	miR-K1	Release of cell cycle arrest	(308)
C/EBPa	Cellular	miR-K3, -K11	Inhibition of lytic gene expression	(309)
C/EBPβ, C/EBPβ p20	Cellular	miR-K11	Induction of IL6/IL10 secretion	(244)
		miR-K3, -K7,		
Ets-1	Cellular	miR-K3, -K11	Inhibition of lytic gene expression	(309)
ICAM1	Cellular	miR-K4-3, -10a	Promotion of angiogenesis	(310)
IKBKE	Cellular	miR-K11	Suppression of interferon signaling	(232)
IRAK1	Cellular	miR-K9	Inhibition of TLR/IL1R signaling	(311)
MAF1	Cellular	miR-K1, -K6-5p, -K11	Induction of endothelial cell reprogramming	(312)
MICB	Cellular	miR-K7	Immune evasion	(246)
MYB	Cellular	miR-K3, -K11	Inhibition of lytic gene expression	(309)
MYD88	Cellular	miR-K5	Inhibition of TLR/IL1R signaling	(311)
NFIB	Cellular	miR-K3	Promote latency	(313)
NFKBIA	Cellular	miR-K1	Activation of NF-κB	(298)
RBL2	Cellular	miR-K4-5p	Inhibition of lytic replication	(314)
ROCK2	Cellular	miR-K4-3, -10a	Promotion of angiogenesis	(310)
SMAD5	Cellular	miR-K11	Inhibition of TGF-β signaling	(304)
TGFBRII	Cellular	miR-K10a, -K10b	Inhibition of TGF-β signaling	(115)
THBS1	Cellular	miR-K1, -K3-3p, -K6-3p, -K11	Promotion of angiogenesis	(315)
TNFRSF10B/TWEAKR	Cellular	miR-K10a	Inhibition of apoptosis	(316)
LANA1	Viral	miR-K10a/b-3p	Regulation of latency	(118)
ORF31	Viral	miR-K3-5p	Regulation of lytic replication	(118)
ORF32	Viral	miR-K3-5p	Regulation of lytic replication	(118)
ORF33	Viral	miR-K3-5p	Regulation of lytic replication	(118)
RTA	Viral	miR-K9*, -K7-5p	Inhibition of lytic replication	(317, 318)
vCyclin	Viral	miR-K10a/b-3p	Regulation of latency	(118)
vFLIP	Viral	miR-K10a/b-3p	Regulation of latency	(118)

counter innate stress response and prevent premature cell death to ensure the completion of lytic replication (50, 53, 101, 210–214). Some of these proteins are expressed at low levels during virus latency, suggesting the possibility that they may also contribute to tumor cell survival. KSHV also manipulates autophagy to counter stress by targeting autophagy protein Beclin1 (215), preventing Atg3 from binding/processing LC3 (207) and blocking Rubicon-mediated autophagosome maturation (216) (Table 3). vFLIP inhibition of autophagy is essential for preventing cells from vCyclin-induced senescence (217).

KSHV also employs several other mechanisms to promote cell survival. For example, KSHV promotes B-cell survival by mimicking an activated B cell receptor (BCR) (218–220). While vFLIP was first described as a dominant-negative inhibitor of Fas-signaling pathways activated during cell-mediated immune killing (221), it is now recognized that it has a more active role in inducing NF-κB signaling, contributing to infected cell survival and proliferation (222, 223). Multiple KSHV miRNAs also activate the NF-κB pathway (208).

Direct KSHV transformation of primary human cells remains elusive. However, a single clone of transformed cells has been isolated from long-term culture of KSHV-infected telomerase-immortalized human umbilical vein endothelial cells (TIVE-LTC) (224). Cell cultures from mouse bone marrow endothelial-lineage cells transfected with a KSHV genome cloned in artificial chromosome (BAC36) (225) can also induce KS-like tumors when subcutaneously inoculated into SCID/NOD mice (226). More recently, it has been shown that KSHV can efficiently infect and transform rat mesenchymal stem cells (227). Subcutaneous inoculation of the KSHV-transformed cells into nude mice induced KS-like tumors expressing markers of lymphatic endothelial, vascular endothelial and mesenchymal precursor cells. The highly efficient nature of this model makes it useful for investigating the functions of KSHV genes in cellular transformation (204, 208).

Immune Evasion

KSHV possesses a number of proteins that target both adaptive and innate immunity (Table 2). Multiple nodes for both type I and type II antiviral interferon signaling are manipulated by KSHV, including interferon-related transcription (101, 228, 229) and the related IRF3, IRF7, and IKKε-proteins (230–234); RNA dependent protein kinase (PKR) signaling (235, 236); interferon-α and -γ receptors (237, 238); and RIG-I activation (239). The inflammasome is induced during KSHV infection (240); however, KSHV blocks NLRP1-dependent innate immune responses (241).

Other components of the innate immune system manipulated by KSHV include ND10-mediated intrinsic immunity (242), PML-mediated transcriptional repression (243), C/EBPβ transcriptional activation of IL-6 and IL-10 (244), NKG2D mediated elimination by natural killer (NK) cells through activation-induced cytidine deaminase (AID) (245), expression of the stress-induced NK cell ligand, MICB (246) and BCR (247), and complement attack during lytic replication (220) (Table 2).

KSHV uses several strategies to prevent efficient viral immune clearance (Table 2). KSHV targets MHC I by inhibiting the processing of viral peptides and down-regulating their plasma membrane presentation to evade CTL surveillance (42, 248–252) and MHC II by inhibiting IRF-4 transactivation of the promoters of CIITA and interferon-γ (253–255). Inhibition of MHC I and II prevents the opportunities for the host immune system to sample viral peptides and blocks initiation of specific CTL responses against latently infected cells (256). During latency, KSHV protein expression is kept to minimal levels to avoid immune recognition, and those proteins that must be expressed to maintain the virus have evolved structural features to inhibit antigen presentation or to mimic host epitopes. KSHV proteins also play a role in inhibiting the cell-surface presentation of costimulatory molecules required for T-cell receptor activation such as B7.2 (257), CD1 involved in NKT cell signaling (258), and the interferon-γ receptor (237). Reduction of cell surface expression of the NKG2D ligands MICA and MICB and the ligand for NKp80, activation-induced C-type lectin (AICL), via its ubiquitin E3 ligase activity, provides a novel way for KSHV to evade NK cell antiviral functions (259). Furthermore, KSHV-secreted proteins, including vIL-6 and three virus-encoded chemokines, may help polarize anti-KSHV immune signaling towards an antibody-predominant Th2 response rather than a cell-mediated Th1 response (260, 261).

LABORATORY DIAGNOSIS

Diagnosis and detection

Direct virus culture from patient specimens is not performed because of the technical limitations in primary isolation of KSHV in cell culture. PCR, however, has been used extensively as a research tool to detect viral DNA in tissues. While PCR detects KSHV DNA sequences in nearly all of the KS tumors examined to date, this technique is of limited use when examining peripheral blood mononuclear cells (PBMC), since only 1 in 50,000 to1,000,000 cells are normally infected (145). Given the small amounts of circulating virus in infected individuals (including those with advanced KS), PBMC preparations are PCR positive only in about one half of patients with KS. Nested PCR, which is plagued by problems of contamination, is frequently required to detect the virus genome under these conditions. Pathologic diagnosis of KSHV-associated diseases from tissue biopsies or cytology is usually straightforward and can be confirmed by immunostaining for LANA1 (Figure 11) (184), a viral protein expressed in every KSHV-infected cell regardless of histogenesis.

Serologic Assays

Serologic assays have reasonable sensitivity and specificity in detecting KSHV infection (Table 6). No assay is currently approved for routine use in patient screening. The most common assays use whole-cell antigen preparations from KSHV-infected PEL cell lines in an indirect immunofluorescence assay (IFA) format (12). These assays are divided into latent or lytic antigen assays depending on whether or not the virus is induced into lytic replication through the use of TPA or another chemical agent. IFA assays, which tend to be labor intensive and require specialized training, are generally being supplanted by ELISA-based assays using recombinant antigens.

Latent-antigen IFAs detect antibodies directed against the ORF73-encoded LANA1 protein (262, 263). This highly charged nuclear phosphoprotein clusters in discrete nuclear speckles and migrates on immunoblots as a ca. 220 to 230 kiloDalton doublet despite its predicted molecular weight of 150 kilodaltons (130). Assays based on LANA1 IFA use unstimulated whole PEL cells, preferably adhered to glass slides using a cytospin technique, in which the characteristic LANA1 nuclear speckling pattern of staining is

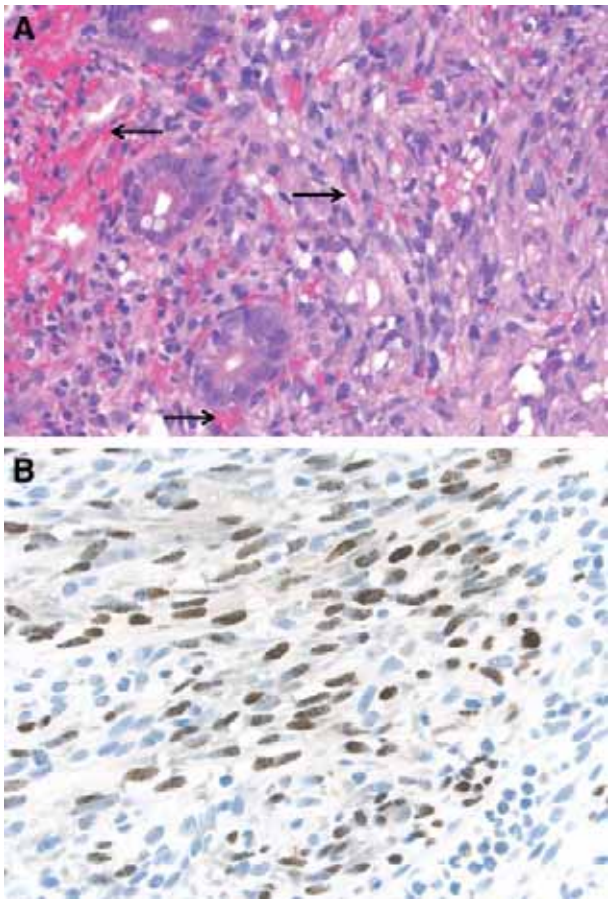

FIGURE 11 A. A photomicrograph of KS tumor infiltrating the duodenum on an AIDS patient. All forms of KS (both HIV+ and HIV−) have similar histologic appearances. Vascular clefts (arrows) within the tumor are filled with red cells, giving the tumor its characteristic reddish-brown appearance. A mononuclear infiltrate in the tumor can be present but cellular atypia or pleomorphism is generally uncommon in KS lesions (×40 magnification). B. Typical speckled nuclear staining pattern (brown) for LANA1 antigen can be seen in many cells of a skin KS tumor (×60 magnification). (Photos courtesy of Liron Pantanowitz, University of Pittsburgh, Pittsburgh, PA.)

present (9, 131). LANA1 positivity is usually determined at a 1:100 or 1:160 serum dilution and extremely high antibody titers (>1:100,000) are not uncommon. This assay gives a 70% to 85% sensitivity rate in experienced hands and is generally highly specific. Generation of recombinant or peptide-mapped LANA1 epitopes tends to reduce assay sensitivity (264). In part this can be overcome by use of baculovirus-prepared antigen, suggesting posttranslational modifications of immunodominant LANA1 epitopes. Immunoblotting for LANA1 gives similar results as does whole-cell IFA but extra effort is needed to efficiently transfer the antigen on gel electrophoresis because of its high molecular weight (130).

Lytic-antigen IFA assays generally have higher sensitivity than LANA1 assays but lower specificity. PEL cells non-specifically bind low-affinity human antibodies (9) and thus serum dilutions less than 1:40 are likely to lead to a false-positive reaction. When appropriately counterstained, diffuse cytoplasmic staining at 1:40 or greater dilution on lytic antigen IFAs can have sensitivity greater than 95%.

TABLE 6 Serologic antigen assays for detecting KSHV infection

Antigen	Gene	Sensitivity	Specificity	Format
LANA1	ORF73	~80%	High	WB, ELISA
K8.1A, B	K8.1	>90%	Intermediate	WB, ELISA, peptide ELISA
SCIP	ORF65	~80%	Intermediate	WB, ELISA
Whole cell (latent)	—	80%	High	IFA, WB
Whole cell (lytic)	—	>95%	Low	IFA, WB

Given concerns about antigenic cross-reactivity in the lytic IFA, considerable effort has been put into development of recombinant lytic protein antigens. Most recombinant assays rely on two proteins, a viral glycoprotein similar to EBV gp220/350 that is encoded by K8.1 and a small capsomere protein, SCIP, encoded by ORF65 (104, 106, 265). Epitope mapping of both proteins has been performed and peptide ELISAs have been developed which work well, particularly for the K8.1 peptide (105, 266). Some cross-reactivity of the amino terminus for KSHV ORF65 protein occurs with EBV hyperimmune sera but this can largely be avoided by using either a truncated C-terminus fragment or a synthetic peptide epitope.

Use of recombinant antigen ELISA has distinct advantages in terms of cost, reproducibility and ease of use. Individual sensitivities for these assays tend to be less than those of whole cell IFAs, but combinations of recombinant antigen assays (including a recombinant LANA1 fragment assay) can be used to achieve greater than 90% sensitivity (267). A combined three-antigen ELISA algorithm using K8.1 and ORF65 peptides together with recombinant LANA1 has been optimized to have ~93% sensitivity and 95% specificity (107). As in the diagnosis of other virus infections, recombinant antigen ELISAs are often used for screening followed by immunoblot assay confirmation. Antibody responses to KSHV tend to be rapid and persistent (Figure 12).

LANA1 antibodies often arise prior to ORF65 protein antibodies (particularly among HIV-infected persons) although exceptions to this are frequent (267, 268). Both LANA1 and ORF65 protein antibodies persist for years after infection and have stable titers throughout the asymptomatic period of infection (9, 267). Antibody titers may increase immediately prior to the onset of KS tumor formation, perhaps reflecting increased viremia concurrent with loss of immunologic control of the virus. However, the variability in absolute virus antibody titer in different individuals limits the usefulness of following antibody titers as a means of predicting onset of disease unless other risk factors are also taken into consideration (123).

For about 20% of HIV-positive persons, antibodies against LANA1 fail to develop at any time during the course of infection. In addition, seroreversion can occur during late-stage AIDS even in persons with florid KS (130). In contrast, persons with HIV-negative KS tend to develop robust humoral immune responses. When responses of HIV-negative KS patients are used as a "gold standard," the sensitivity of some assays may approach 100%, suggesting that assay performance may be affected by the types of populations for which it is used. This has been confirmed by studies showing

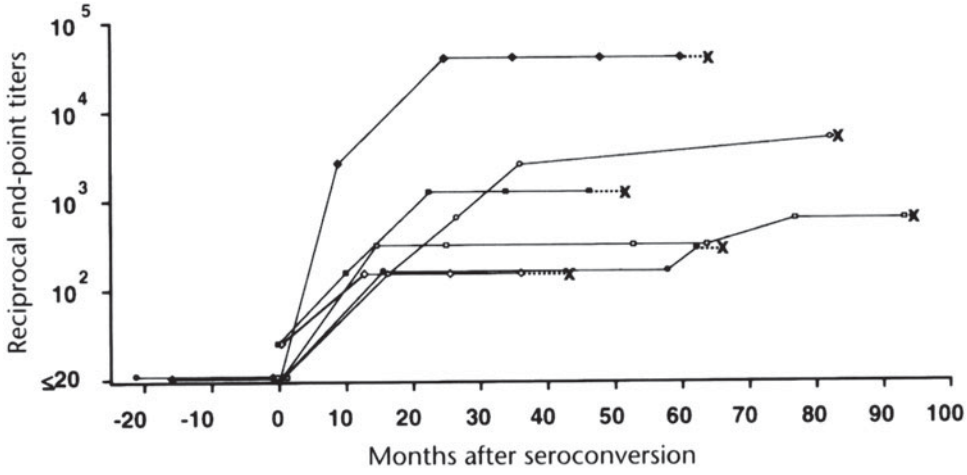

FIGURE 12 Antibody responses to KSHV infection are persistent for years after initial infection. This graph shows the reciprocal end-point titers for six men with AIDS who seroconverted to LANA1 IFA positivity at time 0. Antibody positivity remained stable for up to 8 years until the patients developed KS (marked with an X). Note that anti-LANA1 titers are plotted on a log scale and in some patients can be positive at 1:50,000 dilution or greater. (From Gao et al., (9) with permission.)

loss of LANA1 reactivity, but not other KSHV antigens, among HIV patients with low CD4 counts (269).

PREVENTION

Since the exact modes of transmission are unknown, practical prevention measures have not been developed. Avoiding deep-kissing or use of saliva as a sexual lubricant seem to be evidence-based, simple, and reasonable recommendations to avoid oropharyngeal spread during sexual activity. Condom use is of unclear benefit for preventing KSHV transmission because of limited shedding in semen but should be encouraged to prevent other sexually transmitted infections. While it is unknown whether safe-sex practices have any practical effect on KSHV transmission, clinicians should counsel both HIV-seropositive and HIV-seronegative patients to engage in safe-sex practices to reduce the risk of acquiring opportunistic pathogens such as KSHV as well as limit the spread of HIV.

Screening guidelines for KSHV in the transplant setting have not yet been formally established. Assays to detect KSHV are not readily available to most clinicians although they can be developed in-house by clinical laboratories with sufficient resources and expertise. Since rejection of otherwise healthy allografts from transplantation has critical clinical repercussions, only assays with high specificity (low false-positive rates) should be considered for screening. At this time, elimination of KSHV-infected allografts from transplantation is not routinely practiced. Knowledge of the infection status of the donor and the recipient may allow the clinician to either consider antiviral prophylaxis or to at least monitor the transplant patient for early signs of KSHV-related disease.

Antibody screening for transplant patients, if available, should be strongly considered, particularly for patients in whom reduction in immunosuppressive therapy would have a fatal outcome (e.g., heart, liver transplants). For these patients, if they are KSHV positive or received a KSHV-positive organ, careful follow-up, antiherpesvirus chemoprevention, and use of sirolimus can be considered.

There is no current development of a vaccine despite the established need in African and transplant populations. Unlike other herpesviruses, KSHV has been naturally lost from many populations and is poorly transmissible in others. This suggests that immunogenic vaccines may be capable of preventing infection and, if effective latent antigens are included, used as therapeutic vaccines once infection is established.

Chemoprevention

Chemoprevention is an important and underappreciated prevention possibility for persons at high risk for KS. *In vitro* assays suggest that cidofovir, foscarnet, and ganciclovir have higher specific activity against KSHV than acyclovir (108, 270, 271). A retrospective epidemiologic study shows that AIDS patients receiving foscarnet for prevention and control of CMV retinitis have a lower incidence of KS occurrence (272). In a prospective, randomized clinical trial AIDS patients receiving either oral or intravenous ganciclovir (plus intraocular ganciclovir implants) had 75% and 93% reductions, respectively, in onset of new KS tumors compared to placebo (273). Similarly, foscarnet treatment used to prevent CMV retinitis can delay or prevent KS recurrence and spread among persons with established KS (274). Thus far, these drugs appear to have little activity once KS is established but may prevent emergence of new tumors.

TREATMENT OF KSHV-ASSOCIATED DISEASES

Kaposi's Sarcoma

No specific antiviral therapy against KSHV has been developed to treat KS. KS is currently treated by surgical excision, intralesional or systemic chemotherapy, and localized irradiation. Localized tumors, particularly tumors in non-immunosuppressed individuals, can be excised or irradiated (up to 40 Gy over 20 fractions) with good outcome. KS is, however, a systemic viral disease and local therapy does not prevent dissemination to other sites. Similarly, intralesional injection with vinblastine (0.2 mg/ml for 0.1 ml/cm2 of tumor tissue) has a high partial response rate after single injection, but tumor regrowth is common. A wide range of chemotherapeutic agents either as single agents or in combination have moderate to good responses as palliative

agents in AIDS-KS. KS is also responsive to systemic interferon-α (1–50 MU/m2) but whether this is due to antitumor, immunomodulatory, or antiviral activity remains to be explored.

Several novel therapies are being investigated (275). In contrast to KS, KSHV MCD has been reported to respond to ganciclovir treatment (276) as well as to rituximab (277, 278). Combined use of zidovudine (AZT) and valganciclovir to target the viral phosphotransferase and thymidine kinase appear to be particularly effective in treating MCD (321). In tissue culture cells, nutlin-3a, a reactivator of p53, prevents LANA1 interaction with p53 and MDM2, thereby leading to apoptosis in PEL cells and KSHV-infected endothelial cells (279–281). Sirolimus and similar mTOR inhibitors are effective in controlling classical and transplant KS in contrast to other traditional immunosuppressive agents that induce KS (282). While AIDS-KS patients had partial responses to rapamycin, pharmacokinetic interactions with antiretroviral drugs were observed resulting in a greater than 200-fold escalation of higher doses (283). Molecular studies have identified a number of pathways such as ERK, XBP-1, and ROS hydrogen peroxide that mediate KSHV reactivation. Targeting these pathways might have preventive and therapeutic benefits. In a PEL mouse model, the use of hydrogen peroxide scavenger N-acetyl-L-cysteine (NAC) was sufficient to inhibit KSHV lytic replication, progression of PEL, and significantly prolongs the lifespan of the mice (284).

Among AIDS patients, the most reliable control measure for KS is effective antiretroviral therapy. The rate of new KS diagnoses is markedly lower among persons on highly effective antiretroviral therapy (272, 285). Despite the efficacy of effective antiretroviral therapy, treatment has no apparent effect on long-term KSHV carriage, and as the AIDS population ages there are worrisome reports of re-emergence of KS among patients with low HIV loads and high CD4+ counts (1, 122).

Multicentric Castleman's Disease

Treatment for HIV and KSHV coinfected MCD is not standardized at present; however anti-CD-20 (rituximab) alone or in combination with chemotherapeutics (etoposide, vinblastine, cyclophosphamide, chlorambucil, doxorubicin, and vincristine) appears to confer symptomatic and survival benefits (181, 286). Although anti-CD-20 treatment of MCD may lower the risks for subsequent development of lymphoma, it can perversely exacerbate flares of KS disease and HLH in some patients (287). In a treatment strategy based on the targeting of the IL-6-driven pathogenesis of MCD, human anti-IL-6 antibody (siltuximab) has been demonstrated to attain a significant level of durable tumor regression and symptomatic response for MCD in HIV and KSHV negative individuals (288).

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Adenoviruses

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Adenoviruses were first isolated in 1953 from adenoids and tonsils surgically removed from children (1). Soon after the recovery of adenoviruses from patients with respiratory illness, their role as a major cause of febrile infections in young children and in army recruits was recognized. The illness was originally called acute respiratory disease (or ARD), but the signs and symptoms are similar to those of other viral respiratory syndromes, which should replace the old nonspecific expression.

Adenoviruses are most closely linked with infections of the respiratory tract and conjunctivae and account for 5 to 8% of all pediatric respiratory illnesses. They are also an important cause of childhood diarrhea and have also been implicated in causing myocarditis, encephalitis, aseptic meningitis, hepatitis, and hemorrhagic cystitis. Adenoviruses are increasingly recognized as an important cause of morbidity and mortality in stem cell and solid-organ transplant recipients (2–6).

An effective oral vaccine for adenovirus types 4 and 7 was used in US basic military training populations from 1971 to 1997. During the break in production, adenovirus infections reemerged as the major cause of morbidity. After reintroduction of the vaccine in 2012, marked reductions in adenovirus-related illnesses occurred (7–9).

VIROLOGY

Classification

Adenoviruses are widespread in nature and have been isolated from a large number of species, including fish (*Ichtadenovirus*), amphibians (*Siadenovirus*), reptiles (*Atadenovirus*), and the two most-studied genera, those isolated from primates (*Mastadenovirus*) and birds (*Aviadenovirus*) (10). There is no common antigenic determinant that characterizes the whole family. However, all members of the Adenoviridae family have virus particles of similar sizes, structures and polypeptide compositions.

The human adenoviruses are a rapidly expanding class of viruses comprising more than 54 distinct serotypes that are grouped into seven species (A to G) originally based on serology, hemagglutination properties, oncogenic potential in rodents, and other biological characteristics. The species B adenoviruses are further divided based on tropism and other criteria (Table 1). Members within each species are traditionally subdivided into serotypes based on immunologic differences. Antibodies directed against the two major capsid proteins, hexon and fiber, are the most important determinants in this classification. The genetic relatedness of serotypes within each species is high, and members of each species generally share greater than 85% DNA sequence homology, whereas serotypes belonging to different species show less than 20% homology (11). With the development of new rapid whole genome sequencing methods, the number of adenovirus types is rapidly increasing, the current number being 67. Adenovirus isolates, characterized and numbered based on genomic sequencing data, are often referred to as genotypes, whereas serotypes are reserved for strains classified by serological methods (12). Since the method of typing does not change the numbering, this chapter applies the widely accepted term "type."

Virus Composition

Adenoviruses are nonenveloped icosahedral (20 triangular surfaces and 12 vertices) double-stranded DNA viruses with a diameter of approximately 95 nanometers. The three-dimensional structure of the approximately 150-megadalton (MDa) adenovirus particle has been determined using atomic resolution by X-ray crystallography and cryoelectron microscopy (13, 14).

Figure 1 summarizes the current view of the arrangement of viral polypeptides in the virion. The capsid consist of 252 major capsomers; 240 hexons form the facets of the icosahedron, and 12 pentons, which are located at the corners of the virus particle. The hexon capsomer is a trimer of the hexon polypeptide held tightly together by noncovalent interactions. The pentons consists of two distinct structural entities: the penton base, which anchors the pentons to the capsid, and the fiber, which forms an elongated structure protruding from the vertices. The fiber is a trimer of polypeptide IV, and the penton base is a pentamer of polypeptide III. Adenoviruses belonging to different subgroups have fibers of different lengths and flexibility. The fiber shaft is built up from an approximately 15-amino-acid repeating motif. Hence, the length difference of the fibers results from a difference in the number of repeat units in the fiber shaft. The viral capsid consists of four additional minor capsid protein components, polypeptides IIIa, VI, VIII, and IX, whose location is graphically illustrated in Figure 1. Polypeptide VI binds to an inner cavity of the hexon capsomer

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TABLE 1 Classification of 54 types of human adenoviruses[a]

Species	Type	Site of infection	Receptor	Tumor in animals
A	12, 18, 31	Intestine, respiratory	CAR	High
B1	16, 21, 34, 35, 50	Respiratory, ocular, urinary tract	CD46	Weak
B2	3, 7, 14		Desmoglein-2	Weak
B3	11		CD46/DSG-2	Weak
C	1, 2, 5, 6	Upper respiratory	CAR	Low
D	8–10, 13, 15, 17, 19, 20, 22–30, 32, 33, 36–39, 42–49, 51, 53, 54	Ocular, intestine	CAR Sialic acid (types 8, 19, 37)	Low
E	4	Respiratory	CAR	Low
F	40, 41	Intestine	CAR	Low
G	52	Intestine	Unknown	Low

[a]Adapted from (262) and updated according to (19).

and provides a link between the inside surface of the capsid and the viral DNA. Polypeptide VI is located underneath the peripentonal hexons and polypeptide VIII under the facets. Polypeptide VIII and polypeptide IX stabilizes capsid facets by forming interactions with neighboring hexon capsomers on the inside (pVIII) and the outside (pIX) of the capsid, respectively. Polypeptide IIIa is located at the inner capsid surface as a ring underneath each vertex region. The viral core consists of four proteins, polypeptides V, VII, X (μ), and TP (terminal protein). In contrast to most DNA viruses, adenovirus codes for its own basic histone-like proteins, V, VII, and μ, that complex to the viral DNA within the virion. In addition, the termini of the viral DNA are covalently linked to the terminal protein (TP), which functions as a protein primer during viral DNA replication. For a more extensive review on virus structure (15).

Viral Genome

The viral genome consists of a linear dsDNA molecule with a length of approximately 30,000 to 38,000 base pairs. With the development of new rapid sequencing technologies, the entire sequences for a large number of human and animal adenovirus types have been established (see GeneBank). The lytic replication cycle is divided into two distinct phases: an early phase preceding viral DNA replication during which mainly regulatory proteins are expressed, and a late phase that follows DNA replication and is characterized by the expression of the structural proteins of the viral capsid. The viral DNA encodes for 10 different RNA transcription units, 6 that are active early after infection (E1A, E1B E2A, E3, E4, and L1) and 4 that become activated at intermediate (pIX, IVa2, and L4P) and late (major late transcription unit) times of infection (Fig. 2). All transcription units, except pIX and IVa2, maturate as a complex set of alternatively spliced mRNAs that codes for multiple proteins, many that have distinct biological activities (see below). In addition, adenoviruses encode for at least one (usually two) highly structured small RNAs, the so-called virus-associated (VA) RNAI and VA RNAII (16) that perform important functions during the lytic infection cycle by interfering with the interferon response pathway and the RNAi/miRNA machinery in cells (17).

Biology

The replication cycle of adenovirus is summarized in Figure 3. This cycle takes approximately 30 hours in cultured cells and results in the production of approximately 50,000 to 100,000 new virus particles per cell. Here we will divide the replication cycle into four stages: entry, early events, late events, and virus assembly.

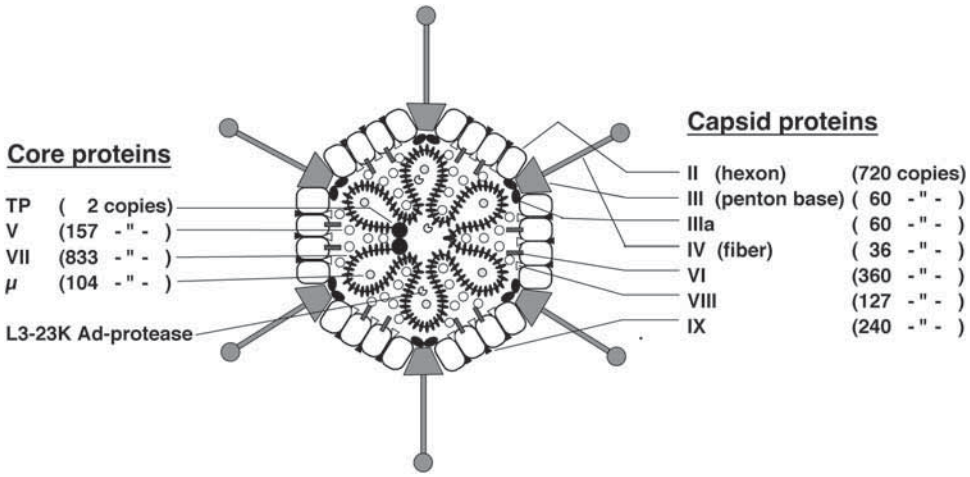

FIGURE 1 Schematic model illustrating the architecture of the adenovirus virion. The current view of the location and the copy number of polypeptides are indicated for the core and capsid proteins. Adapted and updated from (263).

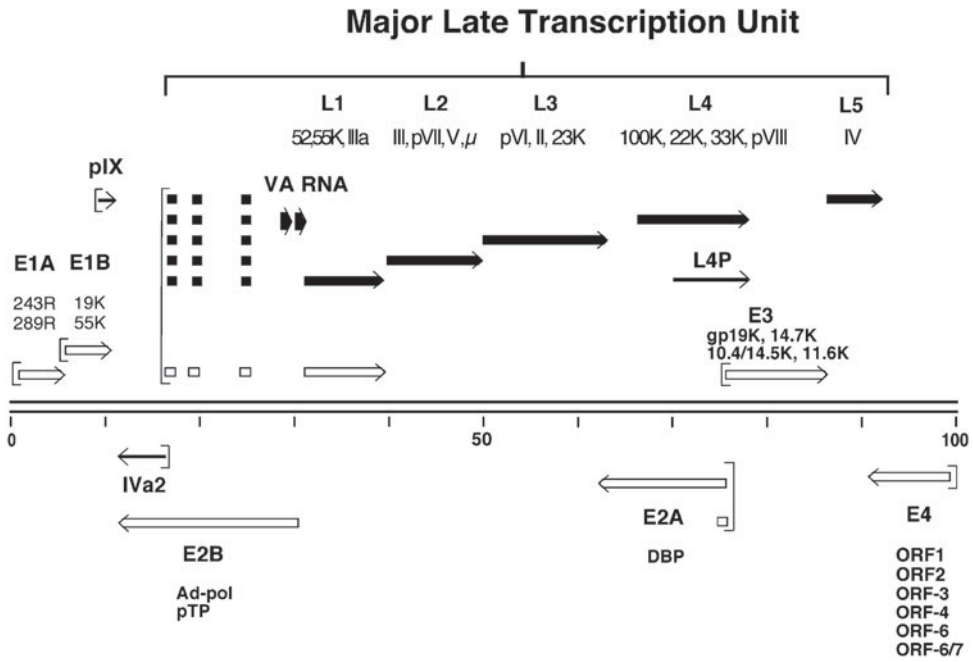

FIGURE 2 Adenovirus transcription map here exemplified with human adenovirus type 2. Note that the alternatively spliced structure of individual mRNAs expressed from the different early and late units are not shown (42). White arrows indicate early transcription units, thin arrows intermediate transcription units and black arrows late transcription units.

Entry

Adenovirus binds with high efficiency to two receptor types on the surface of the host cell; the primary receptor interacts with the carboxy-terminal knob region of the fiber, whereas the secondary receptor interacts with an RGD motif on the penton-base protein. The cellular receptors mediating the initial binding of adenovirus to the cell surface has been identified for several types (Table 1). The best-studied adenovirus receptor is CAR, an immunoglobulin superfamily member-protein, containing two immunoglobulin-like extracellular domains, that is also used by Coxsackie B virus for infection (18). CAR appears to be a preferred primary

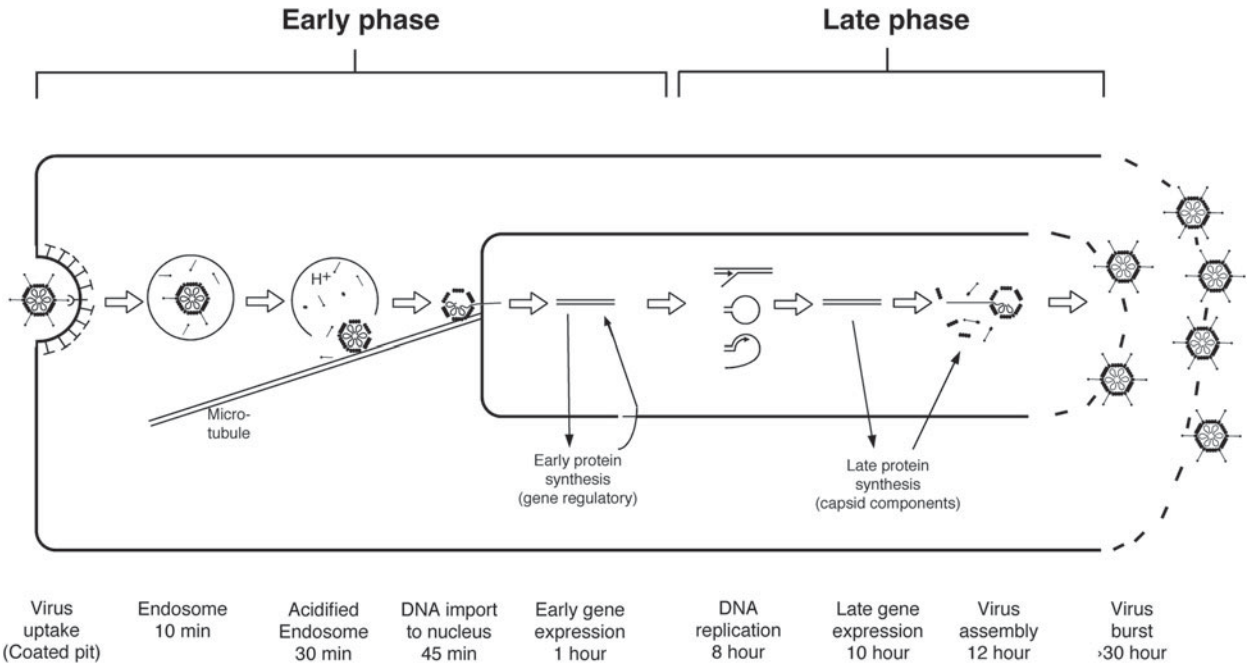

FIGURE 3 Diagram of adenovirus replication cycle. The approximate time scale for the different steps in the adenovirus life cycle is indicated at the bottom of the figure.

receptor used by most types across species, except the species B viruses. In general, species B1 adenoviruses use the complement regulatory protein, CD46, for virus attachment, whereas species B2 viruses use desmoglein-2 as the high-affinity receptor (19). Also, some members of species D, which are associated with epidemic keratoconjunctivitis, use sialic acid as the primary receptor (20). Interestingly, adenovirus infects many cell types efficiently *in vitro*, while adenovirus infection *in vivo* of ciliated lung epithelia is relatively inefficient. This inefficiency appears to result from the fact that CAR plays a role in maintaining the integrity of tight junctions and therefore is sequestered on the basolateral side of polarized epithelial cells (21). Of interest, a single CAR isoform, which is translated from an alternatively spliced mRNA, traffics to the apical surface and is likely to mediate the initial binding of the virus to the airway epithelial cells (22).

Cellular integrins function as the secondary receptor promoting internalization of the virus, most often via clathrin-mediated endocytosis (23). With the exception of the species F viruses, Ad40 and Ad41, all characterized adenovirus types encode for a penton-base protein with an arginine, glycine, aspartic acid (RGD) peptide motif, which mediates the contact with the cellular αβ integrin adhesion receptors. In addition to the first-discovered avß3 and avß5 integrins, additional RGD binding integrins have been characterized (24). Penton-base interaction with the integrins facilitates adenovirus internalization by activating the mitogen-activated protein kinase (MAPK) and phosphoinoside-3-kinase (PI3) signaling pathways, which cause actin cytoskeleton rearrangement (25, 26). The vertex capsomer, the penton, is lost in the acidic endosome (Fig. 3). Following this opening of the capsid, polypeptide VI manages to escape from the interior of the virus particle. Polypeptide VI has a highly conserved amino-terminal amphipathic helix that tethers polypeptide VI to the endosomal membrane and case membrane rupture (27, 28). After escape from the endosome, the partially dismantled virus is transported by the microtubule-associated kinesin-1 motor protein (29) to the nuclear membrane, where it docks to a nuclear pore complex (30). Here the final disassembly occurs to promote importation of the viral DNA into the nucleus. Virus entry is a highly efficient process with 40% of the particles bound to the cell surface delivering their DNA to the nucleus. Less than 5% of the incoming viruses fails to escape from the endosome and ends up in lysosomes for degradation (31).

Early Events

Expression of the early viral mRNAs and proteins are temporally regulated during the infectious cycle. Thus, the first transcription unit to be activated is the E1A region, and transcripts from this region can be detected within 45 minutes after infection. The maximum rate of E1A transcription is then maintained for at least 16 hours. The E1A proteins are essential for the initiation of the lytic infectious cycle, since they are key regulators of both viral and cellular transcription and necessary for efficient S-phase entry of the infected cell (32, 33). E1A encodes for two major protein isoforms, E1A-289R and E1A-243R. The E1A-289R protein is the major transcriptional activator protein and is responsible for the activation the early viral transcription units. E1A-289R activates transcription by recruiting the MED23 mediator subunit to the early viral promoters. E1A is not absolutely essential for early viral gene transcription. Thus, each early unit binds cellular transcription factors that promote a low level of viral transcription. However, at low multiplicities of infection early gene expression needs to be boosted by the E1A-289R protein to initiate effective viral replication. Both the E1A-243R and E1A-289R proteins have the capacity to active the cell cycle by binding the retinoblastoma (RB) family of proteins thereby derepressing E2F transcription factors, which are required for expression of genes required for cell cycle progression. The E1A proteins also sequester the cellular p300/CBP proteins, thereby contributing to the cell proliferative effect of E1A (33). The p300 and CBP proteins are considered as global transcriptional activator proteins that stimulate transcription by inducing histone H3 acetylation. Therefore, the sequestration of p300/CBP by E1A results, on many promoters, in a repression of cellular transcription.

Transcription from regions E1B, E3, and E4 begins around 1.5 hours postinfection. The E1B region encodes for two major proteins, the E1B-55K and the E1B-19K proteins (34). Both proteins serve key functions as suppressors of apoptosis in the virus-infected cell at multiple levels. E1B-55K binds to the activation domain of the p53 transcriptional activator protein, thereby converting p53 from an activator to a repressor of pro-apoptotic genes (32). The viral E1B-55K and E4-ORF6 proteins form a complex with several cellular proteins to form a Cullin5-based ubiquitin-ligase complex. This complex targets the p53 protein for ubiquitination and proteasomal degradation (35). The E1B-55K protein is the substrate-binding protein, whereas the E4-ORF6 protein mediates the contact with the ubiquitin complex. The E1B-55K/E4-ORF6 ubiquitin-ligase complex has several substrates, of which p53 and the cellular MRN complex are the best studied (32). The MRN complex is involved in DNA double-strand break repair by initiating the process of nonhomologous end-joining (NHEJ). The MRN complex needs to be inactivated in adenovirus-infected cells, since the NHEJ proteins disturb viral DNA replication by ligating the linear adenoviral DNA into long concatemers (36). The E1B-19K protein is a homolog of the cellular BCL-2 protein and inhibits apoptosis by sequestering the pro-apoptotic BAK and BAX in inactive complexes (34). In the absence of the E1B-19K protein, an adenovirus infection would cause the BAK and BAX proteins to oligomerize and form pores in the outer mitochondrial membrane, which would result in a release of apoptogenic proteins to the cytoplasm (34).

The E3 region encodes for proteins that counteract the antiviral defense mechanisms of host cells (37). The E3-gp19K glycoprotein protects the virus-infected cell from cytotoxic T-cell lysis by downregulating cell-surface expression of class I major histocompatibility complex (MHC) proteins. E3-gp19K binds and retains MHC in the endoplasmic reticulum, thereby reducing peptide presentation at the cell surface. The E3-14.7K protein inhibits TNF-α-induced apoptosis and the E3-10.4K/14.5K protein complex (the RID complex) inhibits apoptosis by internalizing and degrading the Fas-L and TNF-related apoptosis-inducing ligand (TRAIL) receptors. In contrast, the E3-11.6K adenovirus death protein (or ADP) has a pro-apoptotic activity and participates in the release of the virus after completion of the replicative state of the virus (38).

The E4 region encodes for six proteins, which have been named as open reading frames (ORFs), E4-ORF1, E4-ORF2, E4-ORF3, E4-ORF4, E4-ORF6, and E4-ORF6/7. The E4 proteins participate in a diverse set of activities during virus infection. The best characterized is the E4-ORF6 protein, which forms a complex with the E1B-55K protein and regulates protein stability, as described above. The E4-ORF6

and the E4-ORF3 proteins appear to serve redundant activities, and both proteins can individually replace the entire E4 region to support virus growth in tissue culture cells (39). The E4-ORF4 protein targets the cellular protein phosphatase IIA (PP2A) and redirects the phosphatase to new substrates in the infected cell. E4-ORF4 contributes to the virus replication cycle by downregulating early viral gene expression and inducing hypophosphorylation of various viral and cellular proteins, for example, transcription and alternative RNA splicing factors.

Region E2 is the last early transcription unit to be activated, and RNA synthesis begins around 3 hours postinfection. The E2 region encodes for the three viral proteins needed for viral DNA replication, the viral DNA polymerase (E2B-Ad-pol), the terminal protein (E2B-pTP), and the single-stranded DNA binding protein (E2A-DBP) (40).

Late Events

The late phase of the replication cycle follows DNA replication, which begins around 8 hours postinfection in cell culture experiments. Adenoviruses use an unusual single-stranded DNA displacement strategy to replicate its DNA (40). Initiation of viral DNA replication requires origin sequences that are physically located within the approximately 100-base-pair long inverted terminal repeats located at both ends of the viral chromosome. Replication initiates at either the left or right origin sequence and results in the displacement of the complementary strand as a single-stranded molecule (Fig. 3). Because of the inverted terminal repeat sequences, the displaced single-stranded DNA molecule can form partial duplex DNA structures that can be used for initiation of DNA synthesis of the second strand. Initiation of adenovirus DNA replication occurs by a protein-priming mechanism, where the E2B-pTP, covalently attached to dCMP, the first nucleotide of the nascent strand, functions as primer for the virus-encoded DNA polymerase (E2B-Ad-pol). Elongation is rapid and processive and requires the viral single-stranded DNA binding protein (DBP), which binds to the displaced single-stranded DNA. Only about 10% of viral DNA is incorporated into new virions. Late after infection transcription initiates predominantly at the so-called adenovirus major late-promoter. Transcription from this promoter accounts for more than 30% of total RNA synthesis at late times of infection. This does not necessarily mean that the major late-promoter is a strong promoter, since efficient transcription requires viral DNA replication, which amplifies the number of DNA templates available for transcription initiation (41). The major late transcription unit generates five families of late mRNAs with coterminal 3′-ends (L1-L5; Fig. 2). Following selection of the poly(A) site, a complex set of mature mRNAs are generated by alternative RNA splicing (42). Most of the major late mRNAs encode for structural proteins of the virion (42). A few mRNAs encode proteins that are nonstructural. For example, the L3-23K protease is required for proteolytic trimming of some capsid components late during virus maturation (43). The L4-100K protein is required for late viral protein synthesis and hexon trimer assembly and nuclear transport (44). The L4-22K and L4-33K proteins appear to be the key viral proteins regulating the accumulation of alternatively spliced late mRNAs (45). In addition, the L4-22K and the L1-52,55K proteins are required for viral DNA encapsidation (46, 47). Late during infection, large amounts of the adenovirus VA RNAs (VA RNAI and VA RNAII) are synthesized. VA RNAI protects virus-infected cells against the antiviral effect of interferon by binding to, and preventing activation of, the interferon-induced eIF2a-protein kinase (PKR) (17). In its active form, PKR would otherwise phosphorylate translation initiation factor eIF2, which would result in a general block of translation in virus-infected cells. The noncoding VA RNAs target the cellular RNAi/miRNA pathway and saturate the nuclear exportin 5 receptor and the cytoplasmic Dicer enzyme, thereby interfering with cellular miRNA biogenesis (17). Further, the VA RNAs are cleaved by Dicer into viral miRNAs (so-called mivaRNAs) that are incorporated into functional RNA-induced silencing complexes (RISC).

Late during infection cellular protein synthesis diminishes, resulting in almost exclusive translation of late viral proteins. Mechanistically this occurs by selective transport of late viral mRNAs to the cytoplasm, combined with an almost exclusive translation of mRNAs derived from the major late transcription unit late after infection. The rate of transcription of cellular genes is not inhibited, but nuclear to cytoplasmic transport of cellular mRNAs is strongly reduced in late virus-infected cells (48). Despite this inhibition of transport, viral mRNAs account for only 20% of the total cytoplasmic pool of mRNA. Nevertheless, more than 90% of total protein synthesis in late-infected cells is virus-specific. The mRNAs expressed from the major late transcription unit possess the unique ability to be translated independent of the normal cap-recognition process. During the late stage of infection, the early viral mRNAs and cellular mRNAs are not translated efficiently. The reason for the selective translation of the late mRNAs stems from the fact that all mRNAs produced from the major late transcription unit carry an identical long untranslated 5′ sequence of approximately 200 nucleotides, the so-called tripartite leader. This sequence functions as translational enhancer and allows an mRNA to be translated in the absence of a functional cap-recognition complex. Inhibition of host protein synthesis has been shown to result from a virus-mediated block in cap-binding complex phosphorylation. The viral L4-100K protein blocks the association of the Mnk1 protein kinase that is responsible for phosphorylation of the eIF4E component of the cap-binding complex (49). Under these conditions the L4-100K protein binds to the tripartite leader and recruits the cap-binding complex selectively to the viral late mRNAs. Hence, a selective recruitment of the ribosome-to-tripartite leader-containing mRNAs has been shown to be crucial for the cap-independent translation of viral mRNAs in the late phase of infection.

Virus Assembly

Most viral polypeptides are rapidly transported to the cell nucleus after synthesis. The early first step in virus assembly involves the formation of capsomers from monomeric polypeptide subunits: hexon, penton base, and fiber capsomers. The penton base and the fiber are then combined to form the penton, the vertex capsomer. Formation of the hexon capsomer requires the viral L4-100K protein, whereas fiber trimerization appears to be a spontaneous process. Structural components are made in huge excess in late virus-infected cells. Only 20% of hexon capsomers are incorporated into virions, and only 10% of viral DNA is encapsidated. Despite this wasteful process, as many as 100,000 virus particles are produced per infected cell (50).

The first step in the assembly process is the formation of so-called empty capsids. These appear to contain specific scaffolding proteins and the structural proteins of the virion, except for the core proteins and viral DNA. Empty capsids, which have a light density, mature into heavy intermediates

by encapsidation of increasing amounts of the viral DNA. A cis-acting packaging element has been localized at the left end of the viral DNA, immediately downstream of the left inverted terminal repeat (51). This sequence element causes a preferential encapsidation of viral DNA into an empty capsid beginning with the left end of the viral chromosome. The nonstructural virus encoded IVa2 protein has been implicated as a key ATP driven motor protein that drives the encapsidation of the viral DNA. IVa2 appears to reside at only one unique vertex and, together with the L4-22K protein, binds to the packaging sequence to facilitate the incorporation of the viral DNA into the empty capsid (47, 52, 53). The encapsidation process is complex, and many additional viral proteins have been implicated in the process. After DNA packaging is completed, the young virions mature into fully infectious virions by proteolytic cleavage of six capsid proteins that are made as precursor polypeptides (pIIIa, pVI, pVII, pVIII, pμ, and pTP). This final maturation step is carried out by the adenovirus protease (L3-23K) (43), which is an integral part of the virus particle (Fig. 1). The viral protease requires the viral DNA as a cofactor. The protease has been speculated to use the viral DNA as a guide to slide on, and to reach, all target substrates in the crowded capsid environment (54).

The replication cycle is completed by the release of new virus particles from the infected cell. The adenovirus death protein (ADP or E3-11.6K), which is synthesized at very late stages of infection (more than 20 hours postinfection), promotes cell lysis and appears to be the key factor that ensures efficient release of the virus from infected cells (38).

Experimental Models

Adenovirus prototypes can be propagated in many different continuous cell lines, much of the data on adenovirus replication being produced using HeLa, KB, HEp-2, and A549 cells. Determination of infectious virus titers can be performed in cell cultures by plaque titration assays or immunostaining or a combination of both. Small animal models of adenovirus infection include mouse, Syrian hamster, cotton rat, and woodchuck. Other larger animals like cats and dogs have also been used, while most of the primate experiments have been performed in rhesus monkeys. Because of the large body of research on the use of adenoviruses as therapy vectors, fast molecular methods have been developed to assess the titer and quality of recombinant adenovirus stocks (55).

Inactivation of Environmental Contamination

Nonenveloped adenoviruses with the dsDNA genome are physically and chemically among the most resistant viruses, and water-borne transmission is an important route for epidemic spread of adenovirus infections. Effective water disinfection is achieved with ozone or chlorine treatment (56). For thermal inactivation in liquid, heat-treatment at 70°C or more for 20 minutes or more is recommended (57). Ultraviolet light doses normally used in water treatment are relatively ineffective against adenoviruses, but polychromatic, as compared to standard monochromatic, ultraviolet light shows increased efficiency (58). For surface decontamination, oxidative disinfectants are to be preferred (59).

EPIDEMIOLOGY

Adenovirus infections occur worldwide as causes of endemic and epidemic illnesses. The most common adenovirus types in clinical specimens are the low-numbered respiratory types of species C (types 1, 2, and 5) and B (types 3 and 7), as well as the gastroenteritis types 40 and 41. In the comprehensive WHO epidemiological study from 1967 to 1976, types 2, 1, 7, 3, and 5 (in decreasing order) made up 90% of the 24,184 typed isolates (60). This study did not, however, include types 40 and 41, which could not be isolated during that time. A U.S. study from 2004 to 2006 with 2,237 adenovirus-positive specimens collected from 22 medical facilities found that adenovirus types 3 (35%), 2 (24%), 1 (18%), and 5 (5%) were the most prevalent. In children younger than 7 years, types 1 and 2 were more prevalent than in older children (61). In a recent 4-year study from Finland, types 3 (32%), 4 (22%), 2 (17%), and 1 (9%) were most prevalent among 499 cases; an outbreak of type 3 was evident during autumn of 2010 (Fig. 4) (62). In another recent 8-year prospective study from Spain, types 3, 6, and 5 were most frequent (63).

Since 2003, adenovirus type 14 has emerged and spread throughout the United States causing outbreaks in military training centers and among civilians (64). The causative strain was shown to be a variant type 14 ("14p1"), and the observed virulence was evidently a result of increased transmission in the absence of immunity, rather than increased pathogenicity of the strain (65). The spread of 14p1 has also been reported from Europe and China (66–68). An outbreak of severe pneumonia was associated with 14p1 infections among prisoners in Scotland in 2011 (69).

New types evolve through intertypic recombination within species, especially species D (70). In hospital outbreaks, sequence analysis has been used to confirm the transmission pathways and similarities of strains. For stem cell transplant recipients, molecular typing can be used to discern whether a patient is having a reactivation of a latent adenovirus infection, a nosocomial infection, a community-acquired infection or a donor-associated infection (61).

Incidence and Prevalence of Infection

Many adenovirus infections are subclinical. In large population-based studies, about 50% of persons from whom virus is isolated are asymptomatic (71, 72). However, some of these isolations are made during prolonged excretion of virus in stools after symptomatic infection. Thus, it is possible that the proportion of asymptomatic infections is smaller. One meta-analysis encompassing 1,958 asymptomatic subjects found 5.3% of respiratory specimens positive for adenovirus by PCR (73). Infection caused by gastroenteritis types 40 and 41 is often asymptomatic. In a study of gastroenteritis in Texas day care centers, 46% of such infections were asymptomatic.

Of adenovirus types causing respiratory infections, types 1, 2, 5, and 6 are mostly endemic, whereas 4, 7, 14, and 21 cause epidemics. Type 3 occurs both endemically and epidemically. Outbreaks have been described to occur in closed communities, such as boarding schools and day care centers, and among new military recruits (74). Community-wide epidemics also occur. The types causing epidemic keratoconjunctivitis (types 8, 19, and 37) are often endemic under the poor hygienic conditions of developing countries, but in Western countries, they occur mostly in epidemics which are sometimes nosocomial. The gastroenteritis types 40 and 41 occur endemically throughout the world.

Adenovirus infections elicit neutralizing antibody responses that are type-specific and offer protection against reinfections caused by the same type. Protection is not complete, however, and reinfections may occur. In a Seattle,

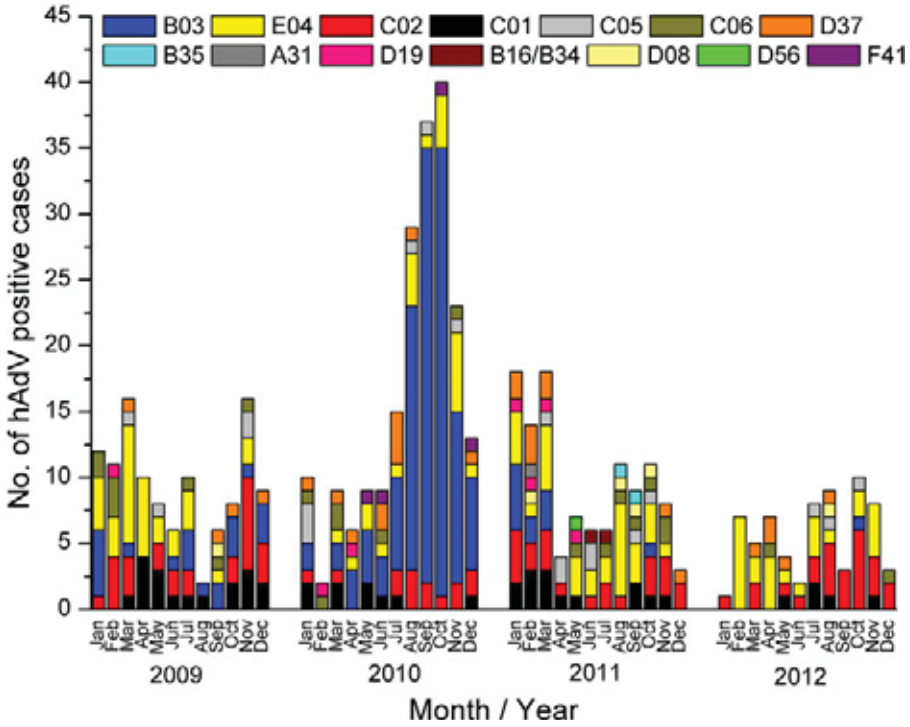

FIGURE 4 The seasonal distribution of adenovirus types circulating in Finland over a period of 4 years. The genotypings performed at the Department of Virology, University of Turku, represent 10 to 23% of adenovirus infections diagnosed in Finland each year. Courtesy of Dr. Minna Ylihärsilä.

WA, virus-watch study, reinfections were observed in 6% of seropositive family members (72); many of these were asymptomatic. In an adenovirus type 3 outbreak in a boys' boarding school, previous infection provided 88% protection against type-specific reinfection (75). Similarly, previous neutralizing antibodies provided 87% protection in staff exposed to the index patient during a hospital epidemic caused by adenovirus type 3a (76).

Age-Specific Attack Rates

Adenovirus infections are most common between 6 months and 5 years of age but continue to occur throughout life. In the metropolitan New York virus-watch study, the incidence of infection per 100 person-years was 40.8 in the age group 0 to 1 years, 33.6 between 2 and 4 years, 15.6 between 5 and 9 years, and 14.4 in the age group 10 years and older (71, 72). These figures concur with seroepidemiological figures showing that about 33% of children have contracted at least one adenovirus infection by the age of 6 to 12 months, and some have already contracted three or four adenovirus infections.

The incidence of enteric adenovirus infections is 4 to 7 per 100 person-years in young children (77). About half of preschool-age children have neutralizing antibodies against these types.

The WHO epidemiological study indicates that types 1, 2, and 5 are commonly contracted during the first years of life; types 3 and 7 are contracted during school years; and some other types, such as 4, 8, and 19, are not contracted until adulthood (60). However, geographical variations exist (78, 79).

High-Risk Groups

Persons with impaired T-cell-mediated immunity have an increased risk of developing severe adenoviral infections. Severe and occasionally fatal disseminated infections have been observed in neonates, in patients with congenital immunodeficiency and in recipients of hematopoietic stem cell transplants. Adenovirus infections are common in human immunodeficiency virus (HIV)-infected patients, but most of these infections are mild or asymptomatic.

Seasonality

In general, adenovirus infections are endemic and detected all year round. Seasonal patterns depend on viral types, population groups, and types of exposure (Fig. 4). Outbreaks of adenoviral respiratory disease are most common in winter and spring (80). Outbreaks of pharyngoconjunctival fever have been associated with swimming pools and occur most often in summer. In the military facilities, adenovirus infections spread when introduced to newly enlisted recruits (81). Adenovirus gastroenteritis shows no distinct seasonal pattern (60, 71).

Transmission

Routes

Adenovirus infections are transmitted by direct contact, by small-droplet aerosols, the fecal-oral route, and sometimes water. The endemic adenoviruses of subgenus C (types 1, 2, and 5), causing childhood respiratory infections, spread by direct contact via respiratory secretions or feces. Self-inoculation with fingers contaminated with infectious secretions is the most important route of transmission (60). For the epidemic types (especially types 4 and 7), respiratory spread by large droplets in close contact and by aerosols is important (81, 82). Aerosol exposure, as measured by adenovirus DNA in ventilation filters, correlated with the number of hospitalizations during an outbreak caused by adenovirus 4 (82). In a military recruit setting, adenovirus DNA was identified in the air and on the surfaces of pillows,

lockers and rifles; virus was also cultured in some samples (81). In hospitals, adenovirus DNA have been frequently found on surfaces like door handles, floors, nurse chair arms, reception desks, and toys, showing that the environment can act as a potential reservoir (83). Types causing pharyngoconjunctival fever and keratoconjunctivitis spread by contact through contaminated fingers or ophthalmologic instruments and also by swimming pool water. The enteric types 40 and 41 spread via the fecal-oral route.

Risk Factors

Close contact in crowded institutions and under low socioeconomic conditions increases the risk for adenovirus infections. Outbreaks have been described to occur in day care centers, schools, hospitals, shipyards, and military quarters. In households, about 50% of susceptible members will become infected after exposure (72). The risk increases with prolonged shedding of the virus. In one family study, 94% of the siblings and 56% of the parents had acute illness during follow-up after exposure; adenovirus disease was confirmed in 63% and 20% of these cases, respectively (84).

Variable attack rates have been observed in closed communities. In an epidemic caused by adenovirus type 3 in a boys' boarding school, the infection rate was 80% among susceptible persons without type-specific neutralizing antibodies, but about half of the infections were subclinical. On the other hand, in an outbreak caused by adenovirus 21 in an isolated Antarctic station, the infection rate was only 15%, although 89% of the personnel were susceptible (85).

Nosocomial Infections

Adenoviruses cause outbreaks of nosocomial respiratory infections. In one outbreak due to adenovirus type 3 in a pediatric long-term care facility, 56% of 63 residents developed adenoviral illness. Seventeen patients (49%) were admitted to intensive care units and two died (86). An epidemic of adenovirus 7a infection in a neonatal nursery causing the death of two patients most likely spread from patient to staff and subsequently to other patients by infected staff (87).

Adenoviruses frequently cause epidemics of keratoconjunctivitis that spread via contaminated fingers, dropper bottles, and improperly disinfected tonometers (88). One outbreak (89) comprised 110 nosocomial cases, and an attack rate of 17% was observed in another (90). An audit study found nosocomial infection rates dropped from 48% to 23% after implementation of infection-control measures, but the rate fell to 3.4% only after patient segregation (91).

Duration of Infectiousness

The median duration of detection of adenovirus DNA in respiratory specimens after acute infection is 11 days (interquartile range 5 to 26 days) in children and 5 days (2 to 20 days) in adults (92). Respiratory adenoviruses, especially types 1 and 2, are excreted in stool for weeks or months after initial infection. Patients are usually considered infectious a few days before the onset of symptoms to approximately 14 days after the onset of symptoms (88). Adenoviruses can remain viable for weeks under proper conditions on common surfaces. With gastroenteritis caused by adenoviruses 40 and 41, fecal excretion lasts 1 to 14 days.

PATHOGENESIS

Incubation Period

The incubation period for adenovirus infection, calculated from index cases or point source exposures, has been calculated to average 7 to 13 days with a range of 2 to 21 days (84, 87, 93–96).

Site of Infection

The primary site of replication of adenovirus is the epithelia of the organs involved. This includes the corneal epithelium, the epithelial lining of the upper and the distal lower respiratory tracts, and the urinary tract (97–100). In contrast, lymphocytes may be the site of chronic persistence of the virus in the nasopharynx (101). The virus may also persist in other tissues, as it can be detected by PCR in 10% of biopsies from patients with various forms of interstitial lung disease not thought to be related to adenovirus infection (102), and in 30% of normal duodenal biopsies (103).

In disseminated infection, adenovirus has also been recovered from blood and from solid organs, including the liver, spleen, kidney, heart, and brain (104, 105).

Histology

Adenovirus pneumonia is characterized by a necrotizing bronchiolitis and alveolitis. The alveolar and bronchiolar cells are enlarged. Alveolar hyaline membranes may be prominent, and there may be extensive alveolar cellular debris. Characteristic cells with basophilic intranuclear inclusions with indistinct nuclear membranes known as "smudge" cells are usually seen (Fig. 5). Other epithelial cells with small eosinophilic nuclear inclusions, amphophilic nuclear inclusions or basophilic inclusions with a clear halo may be seen. An interstitial or alveolar infiltrate is also present. The type of infiltrate may be either neutrophilic, monocytic or lymphocytic or mixed monocytic/lymphocytic (98, 105–107). Based on data from experimental infection, the type of intranuclear inclusions and type of infiltrate likely depend on the duration of infection prior to examination.

Histologic examination of adenovirus ocular infections in humans is limited to visualization of the cornea and conjunctiva *in situ* by live confocal microscopy, as biopsies are not performed.

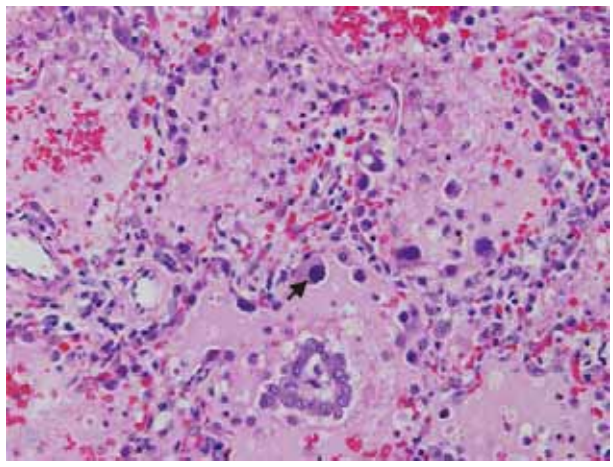

FIGURE 5 Pulmonary histopathology from a patient with a fatal case of adenoviral pneumonia. Characteristic epithelial smudge cells (arrow) show markedly enlarged nuclei containing inclusion bodies surrounded by thin rims of cytoplasm. Inclusion bodies are basophilic or amphophilic when stained with hemotoxylin and eosin. Unlike with herpesvirus infections, no syncytia or multinucleated giant cells are present.

When there is hepatic involvement, fatty necrosis with neutrophilic infiltration or monocytic infiltration has been described, and amphophilic, basophilic intranuclear inclusions and smudge cells are seen (97, 107–109).

Adenovirus colitis occurs in the immunocompromised, particularly in patients with HIV or those post-bone-marrow transplantation. Pathologic findings include characteristic adenovirus epithelial intranuclear inclusions and disorientation of the epithelia with a chronic inflammatory infiltrate. Focal mucosal necrosis has also been described (97, 110–112).

In rare cases of encephalitis, autopsy findings reveal perivascular mononuclear infiltrates, which have also been seen in patients with fatal, disseminated, adenoviral pneumonia with encephalitis (104). In a child with end-stage HIV infection, characteristic intranuclear inclusions were also observed (113).

Virus-Mediated Tissue Damage

There are three mechanisms that are likely responsible for the extensive tissue damage that occurs: 1) direct cytotoxicity due to viral replication or viral components; 2) cytotoxicity due to the inflammatory cell infiltrate; and 3) cytotoxicity due to effects related to pro-inflammatory cytokines stimulated by the virus.

Modulation of Cellular Functions by Adenovirus

During adenovirus replication cycle, the virus profoundly alters normal cellular function, presumably to create conditions that enhance replication. E1A proteins activate cell cycling through subdomain interactions with retinoblastoma protein and p300 (114, 115). Pro-apoptotic effects of E1A are counteracted by several adenovirus proteins including E1B-55K, E4orf6, and E1B 19K, partially by inhibiting nuclear translocation of apoptosis-inducing-factor (116–118). These functions prolong survival of host cells to promote viral replication. However, at late stages in the viral infectious pathway, viral proteins inhibit ongoing cellular processes and viability. E1B-55K and a peptide expressed from the E4 region inhibit nuclear export of cellular mRNAs (119–121). Host mRNA translation is also inhibited (122). Cellular integrity is disturbed through the action of a viral L3 viral protease on the cellular cytokeratin K18 (123). Furthermore, production of the E3 11.6K ADP induces cell death through caspase-dependent and non-capase-dependent pathways (38, 124). These processes may involve modulation of ubiquination of cellular mediators of apoptosis by E1A, E1B-55K, and E4orf4 (125).

Adenovirus cellular structural proteins also interfere with cellular processes. Several of the adenovirus subtypes bind the Coxsackie and adenovirus receptor (CAR). This binding disrupts cellular tight junctions and facilitates virus release (126). The penton base of adenovirus binds to cellular integrins and inhibits cell adhesion (127).

It should be noted that adenovirus replication is not required for inflammation to occur. Wild-type adenoviruses cause inflammation in nonpermissive mouse models (128). Nonreplicating Ad vectors clearly cause significant tissue damage in mice, rats, primates and, unfortunately, in humans (129). This damage likely comes from induction of pro-inflammatory cytokines and recruitment of inflammatory cells (130).

Innate Immune Responses to Adenovirus

Much of the data regarding induction of cytokines by adenovirus is derived from animal models or from studies using nonreplicating adenovirus vectors. In a nonpermissive mouse model exposed to adenovirus intratracheally, TNF-α and IL-6 mRNA were induced in alveolar macrophages but not in epithelial or endothelial cells (131). Intravenous infusion of a nonreplicating adenovirus vector in mice induced TNF-α, IL-6, and IL-12, with tissue macrophages and splenic dendritic cells and macrophages contributing to this response (132). Instillation of WT Ad3p and Ad7h, which cause pneumonia in humans, into a mouse model induces the proinflammatory cytokines IL-1 β, TNF-α, IFN-γ, and IL-12 and the mouse IL-8 homologues, MIP-2 and KC (128). In human monocytes and monocyte-derived macrophages exposed to WT adenovirus type 2, TNF-α was induced in both cell types, but IL-1β was only induced in monocytes (133).

Exposure of human peripheral-blood mononuclear cells to WT adenovirus type 7-induced interferon (not further specified) in culture (134). Blood interferon alfa levels were also elevated in two patients with adenovirus-induced hemolytic uremic syndrome (135).

The source of adenovirus-induced cytokines and chemokines is likely not limited to dendritic cells and macrophages. IL-8 is also induced in pure epithelial cultures by WT adenovirus and adenovirus vectors and in a human lung slice model, where epithelial cells are the predominant cell type (130, 136).

Induction of cytokines during active adenovirus infection in humans appears to be a marker of disease severity. Among 38 children with lower respiratory tract infections with adenovirus, serum TNF-α and IL-6 were detected with higher frequency in patients with severe disease or fatal outcome; IL-8 was detectable in serum in all groups of patients, and levels correlated with disease severity (137). Similarly, in military trainees with febrile respiratory illness due to adenovirus type 55 (14p1), the degree of elevation of serum IL-4, IL-10, IL-15, interferon gamma and interferon alfa-2 levels correlated with either the severity of infection or the presence of symptoms during infection (138). Additional cytokines shown to be induced during adenovirus infection include IL-5, IL-12, IL-17, IL-18, MIP-1, IP-10, and OSM but not MCP-1 or RANTES (139, 140). The cytokine response is likely important in inflammation and cell injury, but it may also be important in limiting dissemination or severity of adenovirus illnesses. Although anti-TNF-α strategies may decrease inflammation during administration of adenovirus vectors to mice, (141) this therapy may predispose humans to severe adenovirus infections (142, 143).

Adaptive Immune Responses

The adaptive immune response is important in preventing reinfection, or, in the case of immunization, illness with adenovirus. Neutralizing and nonneutralizing antibodies to adenovirus are produced during infection. Serologic studies show a high rate of seroconversion. In children with acute adenovirus infection determined by antigen detection or seroconversion, virus-specific IgG antibody increased in 77%, IgM in 48% and IgA in 37%. The IgM titer peaked 10 to 20 days after the onset of illness and remained elevated for two months. IgA titers were variable, decreasing to undetectable levels in some patients and persisting at least 90 days in others (144). In military recruits, IgG levels increased in 89% of the subjects; IgA and IgM titers increased in 77% and 39%, respectively. IgA levels also increase in relevant secretions during adenovirus infections of the nasopharynx, conjunctiva, and intestine (145–147).

The importance of T-cell responses is not well understood, but it is generally regarded that T-cells play a role in

limiting the severity of disease. For example, there is high morbidity and mortality associated with adenovirus infection of patients with transplants, hematologic malignancies or AIDS. Many of these patients are infected with a type of adenovirus, which usually only causes mild disease in immunocompetent patients (148–150). Furthermore, recovery of T-cell counts, decrease in T-cell suppression or infusion of donor lymphocytes decreases viral shedding and the severity of the illness (151).

Both adenovirus-specific CD4+ and CD8+ T-cells are present in normal adults (152, 153). A role for both in containment of adenovirus infection has been proposed. Adenovirus-specific CD4+ T cell clones contain viral replication in infected cell lines and primary bronchial epithelial cells (152). Resolution of viremia is accompanied by the appearance of adenovirus-specific CD4+ and CD8+ T cells (154). Adenovirus-specific CD8+ T cell clones kill transformed lymphoblastoid cells and fibroblasts expressing adenovirus hexon (153, 154).

Adenovirus Persistence and Latency

The classic example of adenovirus persistence dates back to the discovery of the virus. Rowe and colleagues, while trying to develop cell lines from resected human adenoids, noted the development of a cytopathic effect in culture (155). This was due to infection of the cells with adenovirus, which was present in the original subjects in an asymptomatic persistent or latent state. In a similar fashion, viral shedding occurs for months to years after infection, and the half-life of viral DNA persistence in tonsillar and adenoidal tissue has been estimated at 2.6 years (148, 156). Interestingly, more than one strain may persist in the same subject (157).

There are several adenovirus proteins that appear to play a role in evasion of the immune system. This was discovered when infection of cells with adenoviruses with mutations of these proteins increased the sensitivity of lysis of these cells by TNF. These proteins in the E3 region, 10.4K, 14.5K, and 14.7K, act by downstream inhibition of TNF, either through phospholipase A2 or NF-kB (158). Another mechanism that may assist in immune evasion is displayed by E3 19K, which decreases expression of MHC class I molecules in adenovirus-infected cells. Also, adenovirus E1B19K and E3 proteins counteract destruction of infected cells through inhibition of TNF-related apoptosis-inducing ligand (TRAIL), and E3 proteins also inhibit Fas-ligand-induced apoptosis (158, 159). Viral microRNA species are also produced in latently infected cells in culture, and these could facilitate adenovirus persistence by downregulating targeted cellular antiviral mRNAs (160). Additionally, E4orf6/E1B55k protein complexes have ubiquitin ligase activity and degrade specific cellular targets depending on the types of origin. Degradation of these targets, including p53, DNA ligase IV and integrin α 3, has been proposed as a mechanism whereby the virus facilitates the development of latency (161).

These factors are likely responsible for persistence of the adenovirus in human tissues by preventing elimination of virus-infected cells. There has also been speculation that persistence, or true latency with integration of adenovirus genes, plays a role in the development of human illnesses. One study, comparing lung tissue from patients with COPD to patients with similar smoking histories without the disease, found that afflicted patients had increased amounts of adenovirus E1A by PCR and increased detection of the E1A RNA by *in situ* hybridization (162). Follow-up studies by the same group showed that areas of increased E1A expression in alveolar epithelia correlated with cellular inflammation and severe emphysema (163). This issue has not been settled, however, as a subsequent study has shown a low incidence of E1A RNA expression and E1A DNA in patients with COPD (164).

In a study of adenovirus-persistence in children, adenovirus capsid in BAL fluid from children with treatment-resistant asthma was detected at high rates compared with children of similar age without asthma (165). This finding should be tempered by the fact that others have shown that steroid treatment of patients with interstitial lung disease increases the detection of adenovirus by PCR (102).

There are several cross-sectional studies suggesting that adenovirus type 36, and possibly type 5, infections, as determined by the presence of antibodies to these strains, are associated with obesity (166). A meta-analysis of 11 of these studies showed a 1.6 odds ratio (95% CI=1.14–2.25; $P<0.01$) for obesity for subjects seropositive for adenovirus type 36 and that the odds ratio was increased to 1.95 in children (95% CI=1.34–2.85; $P<0.01$)(167). The role of adenovirus 36 as a causative agent of obesity remains controversial because there have been negative studies and because few longitudinal studies have been performed (168). In any case, these studies raise provocative questions about the possible role of persistent or latent adenovirus in the development of chronic respiratory diseases and other illnesses.

CLINICAL MANIFESTATIONS

Acute Respiratory Infection

Adenovirus infections most commonly occur in children between 6 months and 5 years of age and manifest as febrile upper respiratory tract infections. Asymptomatic infections are common and most symptomatic infections are mild and self-limited. Adenoviral infections cannot be distinguished clinically from infections induced by other respiratory viruses (72, 80, 169). In one 12-month study, adenovirus was identified by PCR in 3% of 543 acute respiratory illnesses; the mean duration of adenovirus illness was 18.6 days. Importantly, another respiratory virus is present in up to 60% of adenovirus-positive cases (63,170–172).

Clinical syndromes during adenovirus infections include tonsillitis, pneumonia, acute otitis media, conjunctivitis, febrile convulsions, fever without focus of infections, and laryngitis (173–175). High and persistent fever is common in children. In hospitalized children with adenovirus infection, the mean maximal temperature was 39.4°C, and the mean duration of fever was 5.4 days (range, 2 to 13 days) (174, 176).

About half of children with adenovirus infection have a marked leukocytosis ($>15{,}000/mm^3$), elevated erythrocyte sedimentation rate (>30 mm/h), and elevated serum C-reactive protein levels (>40 mg/liter), in contrast to most cases of influenza virus, parainfluenza virus and respiratory syncytial virus-induced illnesses (63,177–179). Except for respiratory syncytial virus infections (in which as much as 60% of cases also manifest acute otitis media), the number of possible bacterial coinfections does not differ with different virus infections. The cause of the elevated laboratory values in adenovirus infections is unknown (177, 178).

Adenoviral infection may mimic a bacterial infection and is a common cause of ineffective and unnecessary antibiotic treatment. High-grade and prolonged fever and common abnormal laboratory findings are the major reasons for antibiotic treatment in clinical practice.

Tonsillitis

Tonsillitis is a frequent clinical manifestation of adenovirus infection. Two studies of acute febrile tonsillitis found that adenoviruses were the most common single causative agents and accounted for 19% of cases (180, 181). Adenoviruses and group A streptococci usually do not cause mixed infections in children. In adenoviral tonsillitis, the exudates are most often thin and follicular or netlike but sometimes may have thick membranes (176, 180) (Color Plate). White blood cell (WBC) count, serum C-reactive protein and erythrocyte sedimentation rate do not distinguish bacterial from viral tonsillitis. Although age groups overlap, adenoviral tonsillitis occurs most often in children less than 3 years of age, whereas beta-hemolytic streptococci induce tonsillopharyngitis in 5- to 17-year-old children. Adenovirus infection is a frequent cause of tonsillitis unresponsive to antibiotic therapy and requiring referral to a hospital (144, 181, 182).

Pneumonia

Approximately 10 to 20% of childhood pneumonias and 1 to 4% of pneumonias in adults are attributed to adenoviruses (171, 183, 184). Conversely, pneumonia is a main diagnosis of 4 to 18% of hospitalized children identified with an adenovirus infection (174,185–188). In basic military trainees, adenoviruses have been implicated in 90% of pneumonia-related admissions. Adenoviral pneumonia results primarily from infections with types 4, 7, 21, and 3. These types may also cause disseminated disease.

Adenoviral pneumonia cannot be clinically distinguished from other viral or bacterial pneumonias. Chest roentgenogram findings vary from diffuse to patchy interstitial infiltrates. Consolidation and pleural effusions have been described. Parahilar peribronchial infiltrates and atelectasis occur in most children with abnormal roentgenographic findings associated with adenovirus infection. Adenoviral pneumonia may be associated with disseminated infection involving the heart, liver, pancreas, kidneys, and central nervous system. The fatality rate can be as high as 30% (189). Permanent lung damage after adenoviral pneumonia has been reported to occur in 27 to 65% of those who had adenovirus type 7 pneumonia. These changes include bronchiectasis, bronchiolitis obliterans, and unilateral hyperlucent lung (McLeod) syndrome. Many patients with normal chest roentgenograms have abnormal pulmonary function tests, often consistent with pulmonary obstruction (190). The young age of the child and measles are risk factors predisposing patients to chronic lung damage.

Pharyngoconjunctival Fever and Keratoconjunctivitis

Pharyngoconjunctival fever and keratoconjunctivitis are two well-described adenovirus syndromes affecting the eye. Pharyngoconjunctival fever occurs principally in children and was recently associated with types 8 and 4 (191).

Pharyngoconjunctival fever is often associated with preauricular adenopathy. Conjunctivitis is usually unilateral or asymmetric. In the early stage, adenoviral conjunctivitis cannot be distinguished clinically from bacterial, allergic, or other viral acute conjunctivitis. Manifestations include watering, redness, discomfort, and photophobia, typically lasting 1 to 2 weeks. The palpebral conjunctiva is hyperemic and contains diffuse infiltration and papillary or follicular hypertrophy. In severe cases, subconjunctival hemorrhages, chemosis, or pseudomembranes occur.

Epidemic keratoconjunctivitis occurs mainly in adults and is usually caused by adenovirus types 8, 19, and 37 (60, 192, 193). In 99 children with acute conjunctivitis seen in a pediatric practice, *Haemophilus influenzae* (42%), adenoviruses (20%), and *Streptococcus pneumoniae* (12%) were the most common etiologic agents. Simultaneous infection with two pathogens was uncommon. The clinical features of conjunctivitis by the three different pathogens are summarized in Table 2. Eleven (65%) of the 17 children with both pharyngitis and conjunctivitis had adenovirus cultured from the conjunctiva (194).

A large number of epidemics of adenoviral keratoconjunctivitis have been described. During 2008 to 2010 in the United States, 6 unrelated outbreaks with 411 cases were reported to CDC (88). Outbreaks of keratoconjunctivitis typically occur in industrial plants, ophthalmology clinics, hospitals, nursing homes, camps, military bases, and child care centers. The virus is transmitted by the hands of medical personnel and by contaminated ophthalmic solutions and instruments. In industrial and military base outbreaks, spread may occur by the common use of bathrooms and by inadequate hygiene. Adenoviruses can remain viable for several weeks on wash basins and hand towels. The illness is first characterized by conjunctivitis, chemosis, pain, photophobia, and lacrimation. A diffuse punctate epithelial keratitis occurs within 3 to 4 days. It may resolve within 2 weeks but can develop into focal subepithelial keratitis with pathognomonic corneal opacities. In rare cases, stromal infiltration may persist for months or even years. Fortunately, the illness is usually self-limited, and the patient's vision remains unaffected (192, 193).

Enteric Infection

Adenoviruses are detected in 3 to 14% of stools from children with gastroenteritis in hospitals, outpatient clinics, and day care centers but also in 9 to 42% of asymptomatic children when PCR is used (195–197). Of the adenoviruses detected in the feces, types 40 and 41 comprise 30 to 80%, respectively, in different studies. The other common adenovirus types found in the stools are 1, 2, 3, 5, 7, and 31. Adenoviral diarrhea is most common in children less than 2 years of age. By the age of 3 years, 30 to 100% of children have neutralizing antibodies to adenovirus types 40 and 41.

No special feature clinically differentiates adenoviral diarrhea from diarrheas induced by other viruses (196, 198). The stools are usually watery and nonbloody, in contrast to bacterial diarrheas. Mucus is noted in 19 to 57% of cases. The maximum daily frequency of stools varies from 3 to 15. The mean duration of diarrhea is 3 to 11 days, often being

TABLE 2 Clinical features of conjunctivitis caused by three different pathogens[a]

Clinical feature	*H. influenzae* (n = 42)	*S. pneumoniae* (n = 12)	Adenoviruses (n = 20)
Mean age of patients (yr)	3.6	3.1	8.5
Bilateral disease (%)	74	50	35
Purulent exudate (%)	93	83	45
Concurrent otitis (%)	33	8	10
Concurrent pharyngitis (%)	5	8	55

[a]Modified from reference (194) with permission.

significantly longer than rotavirus-induced diarrhea. In patients seeking care for diarrhea, the severity of adenoviral diarrhea has been similar to that of rotavirus infection, whereas in studies of outpatients, rotaviral disease seemed to be more severe (199). Type 41 adenovirus-induced diarrhea lasts longer than type 40-induced diarrhea (means, 12.2 days versus 8.6 days, respectively). Enteric adenoviruses seem to induce longer enteritis than nonenteric adenoviruses (200). Fever and vomiting in enteric adenovirus infections are common. Although adenoviral gastroenteritis is usually a mild disease, fatal cases in immunocompromised patients have been described.

Viral and bacterial copathogens in adenoviral diarrhea have been found in 13 to 18% of the cases, and other viruses were detected in respiratory specimens from 26% of 47 patients when adenovirus was visualized in the fecal sample (196).

Fever Syndromes

In 105 hospitalized patients with adenovirus infection, 17% had fever with no identifiable site of infection (174). In another study, adenovirus was cultured from 6% of 116 patients with fever without a localized cause (201). Samples for PCR tests from the nasopharynx and feces should be included in the workup of febrile patients without focus of infection.

Adenoviruses are the most common single etiologic agents of febrile convulsions in young children and caused 13% of 144 cases in one study. In three patients, adenovirus was also detected from cerebrospinal fluid, suggesting that febrile convulsions may have been the sole manifestation of adenoviral central nervous system infection (202).

Hemorrhagic Cystitis

Hemorrhagic cystitis is a self-limited illness in otherwise healthy children, occurring more often in boys than girls. Adenovirus type 11 has been recovered from the urine of 19 to 91% of the patients. In addition to gross hematuria, the clinical manifestations included urgency, frequency, and fever. The duration of gross hematuria varies from 2 days to 2 weeks, and there are no changes in serum creatinine levels (203).

Hemorrhagic cystitis associated with adenovirus has been described to occur following bone marrow and renal transplantations. Coinfection with BK polyomavirus and cytomegalovirus may occur. Female sex, seropositivity for adenovirus prior to bone marrow transplant (BMT), and acute graft-versus-host disease are significant risk factors. Studies from the United States reported adenovirus-associated hemorrhagic cystitis after BMT in only 0.3 to 1.0% of recipients (204). In renal transplant recipients, the disease is associated with gross hematuria, urinary frequency, burning urination, fever, and negative bacterial cultures. The symptoms last for 2 to 4 weeks and serum creatinine increases (205).

Infection in Military Recruits

Prior to vaccination, up to 80% of recruits experienced adenovirus infection. Among 58,103 febrile respiratory illnesses in the U.S. military basic trainees, 64% were laboratory-confirmed adenovirus infections (8). Physical and mental stress and crowding are considered major reasons for the susceptibility. The most common clinical manifestations in military recruits mimic those found in children, sore throat, nausea/vomiting, cough, and fever. Adenovirus-associated illness occurred most commonly in training weeks 3 to 6 while influenza occurred in weeks 0 to 2 (206). A large epidemic of keratoconjunctivitis with nearly 3,000 cases occurred at a U.S. military base in the Philippines, and this event led to recommendations for management of the epidemic (Table 3) (207). In one study of 108 young men with febrile tonsillitis in the military service, adenoviruses caused 31% of the cases, whereas group A streptococcal infections were detected in 38% (207). In 17% of cases of streptococcal tonsillitis, evidence of concurrent adenovirus infection was also found, in contrast to adenoviral tonsillitis in children (180).

Adenoviral pneumonia in military conscripts is most often caused by adenovirus types 4 and 7, which are the types included in the oral live vaccine used successfully between 1971 and 1999 and from 2012 onwards in the U.S. military. During vaccine unavailability, severe epidemics of adenovirus 4 infections affecting thousands of trainees were reported, and adenovirus 4 was responsible for nearly all diagnosed cases of adenovirus infections in the U.S. military recruits. Eight deaths were reported during the break of adenovirus vaccination, mostly due to pneumonia (9). From 2002 to 2006, epidemiological studies using molecular diagnostic procedures revealed the emergence of adenovirus type 14 (64). Interestingly, annual cases of adenovirus 14 decreased markedly after the resumption of adenovirus 4 and 7 vaccination (8).

Infection in Newborn Infants

Severe adenovirus infection in newborn infants is rare, but infants are susceptible to a disseminated form of adenovirus infection, which has been fatal in 68% of cases (208). Transmission may occur either vertically, in utero from the infected mother, or horizontally, after birth. Adenovirus outbreaks in neonatal intensive care units have been reported. In vertical infections, mothers often have viral symptoms preceding, or shortly after, delivery, and there may be prolonged rupture of membranes. The illness starts within 10 days of age with lethargy, fever or hypothermia,

TABLE 3 Epidemic keratoconjunctivitis preventive measures[a]

General recommendations
Wash hands thoroughly.
Clean and sterilize instruments.
Use unit doses of ophthalmic solutions.
Examine “red eye” patients in a separate area.
Educate staff about epidemic keratoconjunctivitis and other transmissible eye diseases.
Avoid use of cloth towels in bathrooms.
Termination of an outbreak
Observe above recommendations.
Communicate the existence of an outbreak to all staff.
Segregate patients by presence or absence of epidemic keratoconjunctivitis.
Discard all open ophthalmic solutions.
Remove infected personnel and patients from clinic for 2 wks.
Determine the cause of the outbreak and mode of transmission.
Educate patients about epidemic keratoconjunctivitis.
Postpone elective procedures.
Act with speed and decisiveness.

[a]Adapted from reference (192) with permission of Oxford University Press.

anorexia, apnea, hepatomegaly, bleeding, and progressive pneumonia. Adenovirus types 3, 7, 21, and 30 have been cultured most often from the lungs and liver (209). In horizontally transmitted infections, neonates often have ill contacts. The mean age of the patients in one study was 16 days. Four neonates required extracorporeal membrane oxygenation (ECMO).

Infection in Immunocompromised Patients

Adenovirus infections in immunosuppressed patients are more often disseminated, persistent, and associated with different types of adenoviruses than in healthy hosts. Severe adenovirus infections are increasingly being recognized, especially in recipients of hematopoietic stem cell transplants (HSCT) (2, 150). The incidences of adenovirus DNAemia in pediatric allo-HSCT recipients vary from 6% to 42% and in adult allo-HSCT patients from 3% to 15%, depending on the transplant type and intensity of surveillance (5). Among 2,879 adult allo-HSCT patients between 1999 and 2008, adenovirus infection was diagnosed by viral culture in 73 patients (2.5%), in whom mortality was 22% (210). Among 624 adult and pediatric allo-HSCT patients, adenovirus DNAemia occurred significantly more often in T-cell depleted, compared to conventional, allografts (4% versus 7.9%), and, importantly, the risk for invasive adenovirus disease was 10-fold higher in the T-cell depleted allograft group. Half of the patients with very high adenovirus DNAemia ($>2 \times 10^3$ copies/mL) died (211). One early study, conducting weekly viral surveillance cultures from the throat and stools and also in the urine of adults during the first 100 days after transplant in 201 BMT recipients, found that the incidence of adenovirus isolation was 31% for children and 14% for adults. Thirty-one percent of all adenovirus-positive patients had definite or probable adenoviral disease, representing 6.5% of all patients (212). Most adenovirus infections in children develop within 30 days after transplantation, whereas in adults infection usually develops more than 90 days after transplantation (5). The infection is thought to be secondary to reactivation of persistent or latent viruses or the transmission of latent virus via donor graft. In young children, infection may be acquired from a family contact or nosocomially (213).

The adenovirus types detected from HSCT recipients differ from those detected from otherwise healthy subjects. The most commonly reported adenovirus types include adenovirus C1, C2, B3, B7, B21, A31, E4, and F41. However, typing is not necessary for optimal treatment (5, 214).

According to the 2013 Guidelines from the American Society of Transplantation, adenovirus disease necessitates the presence of attributable organ signs and symptoms combined with virus detection in biopsy specimens on immunohistochemical staining or in bronchoalveolar lavage and cerebrospinal fluid (culture, antigen detection, or PCR), in the absence of another diagnosis. Disseminated adenovirus disease requires involvement of two or more organs, not including viremia (6).

The clinical manifestations of adenovirus infection in HSCT recipients may include high fever, pneumonia, hemorrhagic cystitis, encephalitis, hepatitis, nephritis, and colitis. Adenovirus has been the major contributor to mortality in about one-half of those with definite disease. Most of the fatal outcomes result from fulminant pneumonia, hepatic failure, nephritis, or colitis. Male sex, young age, alemtuzumab treatment, cord transplant, T-cell-depleted grafts, acute graft-versus-host disease, lymphocytopenia ($<300/mm^3$), and detection of adenovirus from multiple sites are clear risk factors for disease (5, 210, 211, 213). Routine PCR tests for detection of adenovirus DNA in nasopharyngeal mucus, feces, conjunctivae, and possibly from lung biopsy samples should be included in the etiologic workup of immunocompromised patients with suspected infection.

Weekly monitoring of blood samples for adenovirus DNA by real-time quantitative PCR has been shown to be useful in recognizing patients at risk for a potentially severe infection (215). In one study of 238 consecutive pediatric allo-HSCT recipients, weekly DNAemia monitoring revealed adenovirus loads greater than 1,000 copies per milliliter in 15.5% of all patients. Adenovirus DNA in blood before day-50 posttransplantation predisposed to development of high adenovirus loads ($>10{,}000$ copies/ml) (214). Increased or increasing viral loads in blood or stool are associated with an increased risk of death, and preemptive antiviral treatment should be considered (2, 213, 215).

Adenovirus DNAemia is also relatively common (5 to 20%) in adult lung, kidney, and heart transplant recipients, but most infections were asymptomatic, and routine screening for adenovirus DNA is not recommended in solid-organ transplant recipients (2, 5). In one study adenovirus was cultured from 10% of over 400 liver transplant patients at a median of 26 days from transplantation, most commonly in urine. Hepatitis was the most common invasive disease (29% of infections), and type 5 caused most cases of hepatitis. All patients had high fever lasting 6 to 44 days. In addition to elevated transaminase levels, the patients had characteristic histopathology in the liver consisting of adenoviral inclusions or microabscesses.

Adenovirus infections occur frequently in HIV-positive patients. The actuarial risk of adenovirus infection at 1 year in late-stage HIV disease was 31 to 38%. Infection occurred most often in the gastrointestinal tract inducing diarrhea only, but half of the patients remained asymptomatic or minimally symptomatic (216). In AIDS patients adenoviruses may be associated with pneumonia, hepatitis, encephalitis, nephritis, and gastroenteritis (150).

Uncommon Clinical Manifestations

Adenoviruses have been infrequently isolated from the cerebrospinal fluid and brains of patients with meningoencephalitis. Often the patients have been neonates or immunocompromised persons with disseminated disease. In one adenovirus type 7 epidemic affecting 32 previously healthy children, 25% had meningoencephalitis, and 10% died. All children also had respiratory symptoms, including pneumonia in six cases (217). In 2,398 children with adenovirus infection, 3.3% had manifestations of central nervous system dysfunction (188, 218). In the California Encephalitis Project, adenovirus was found as a possible cause of encephalitis in about 1% of 1,570 patients studied (219).

Sensitive molecular techniques have suggested that adenoviruses may be causative agents of myocarditis. In three studies, cardiac samples were obtained for PCR analysis from patients with myocarditis and from patients with dilated cardiomyopathy. Adenoviruses were identified in 8 to 23% and 1.6 to 12% of the samples, respectively (220).

Adenovirus infections have been thought to induce mesenteric lymphadenopathy, which then could act as a mechanical lead point for the intussusception. In one study, 47% of 53 children with intussusception had adenovirus in their stools. Of 25 cases, adenovirus group C was most common. Of note, simultaneous infection with HHV-6 and adenovirus carried the highest risk for intussuception (221).

Forty cases of adenovirus urethritis and concurrent conjunctivitis have been reported. Patients typically present with dysuria, clear or mucoid urethral discharge, and evidence of meatitis. Conjunctivitis may be bilateral. The mean duration of this self-limited illness is 14 days. Oral sex is the likely transmission route (222).

Bacterial Complications

Acute otitis media (AOM) occurs in 30 to 37% of children with adenovirus infection, and 3 to 10% of AOM patients have evidence of adenovirus infection. Adenovirus was found in 2% of 581 middle-ear fluid samples from AOM patients (223).

Excluding military conscripts, adenovirus-induced febrile tonsillitis is seldom associated with group A beta-hemolytic streptococci. Similarly bacterial coinfections have been very rare in adenoviral keratoconjunctivitis. Serologic evidence of bacterial infection was found in 47% of 19 children with adenoviral pneumonia (185). Recently, a U.S. study of childhood community-acquired pneumonia reported 248 cases of adenovirus pneumonia, out of which 10% had evidence of bacterial coinfections (171). Approximately one-half of military conscripts with adenoviral pneumonia have evidence of concomitant bacterial infection. One study in a developing country showed that adenovirus lower-respiratory-tract infection is associated with high nasopharyngeal pneumococcal colonization and, further, with invasive pneumococcal pneumonia (224).

LABORATORY DIAGNOSIS

The clinical picture of a respiratory adenovirus infection is variable and commonly resembles that due to other microbes (175). Up to 80% of acute conjunctivitis is caused by viruses, of which 65 to 90% are adenoviruses, but the accuracy of clinical diagnosis is only 50% (225). Therefore, adenovirus infections can seldom be diagnosed on clinical grounds alone, and laboratory testing is needed for specific diagnosis.

Virus Isolation

Specimen Types

Adenoviruses have been isolated from stool, throat swabs, nasopharyngeal aspirates, conjunctival swabs and scrapings, urine, cerebrospinal fluid, blood, and a variety of biopsy specimens. The optimal specimen type depends on the clinical picture and, to some extent, on the adenovirus type in question.

Most adenovirus infections involve viral excretion in stool, which makes it a practical specimen for detection. However, excretion of the respiratory types in stool can continue for several months. Therefore, isolation from stool does not have the same diagnostic significance as isolation from the involved site, for example, the respiratory tract or eye specimens. On the other hand, detection of high adenovirus DNA load in stool of transplant patients may precede viremia by several weeks (226).

Specimens should preferably be collected within a week after onset of illness. After that, in most cases, the excretion of virus and the sensitivity of isolation will decrease. Swab and biopsy specimens should be collected in transport medium. Stool and cerebrospinal fluid specimens can be transported, as such, in clean containers. Adenoviruses are relatively stable. Shipment on ice (4°C) or frozen is preferable for maximum sensitivity, but most adenoviruses can be grown in specimens transported at room temperature.

Cell Culture

Cultures of the appropriate site during active infection have high yields. This includes cultures of nasopharyngeal swabs or aspirates, throat swabs or washes, conjunctival swabs or scrapings, bronchoalveolar lavage fluid, stool or rectal swabs, urine or urethral swabs, cervical swabs, CSF, and tissue samples. Adenoviruses are species-specific, and isolation is best achieved in human cells, although cynomolgus monkey kidney cells can also be used. All adenovirus types, except 40 and 41, grow well and produce cytopathic effect in a variety of human epithelial cells. The best sensitivity is achieved with human embryonic kidney cells, but because these are expensive and difficult to obtain, continuous cell lines are commonly used. Suitable continuous cell lines include A549, HeLa, HEp-2, KB, and MRC-5 strains.

Some strains of the enteric types 40 and 41 will grow in these cell lines, but the best growth is achieved in HEK 293 cells (human embryonic kidney cells transformed by adenovirus type 5 DNA). Because of strain variation in the growth pattern, sensitivity is increased by simultaneous inoculation of the specimens into two or three different cell lines.

Adenoviruses produce typical cytopathic effects (CPE), which often start at the periphery of the monolayer. The cells become rounded, with characteristic refractile intranuclear inclusions. The appearance of CPE is relatively slow, especially in the continuous cell lines. For the best sensitivity, a 4-week incubation with blind passage is recommended. Among healthy children, adenovirus is rarely cultivable ($\leq$1%) from throat swab specimens, and adenovirus isolation can be considered diagnostically significant and indicative of an acute infection (201, 227). Shell vial assay, in which monolayers are tested with monoclonal antibodies to the hexon protein, can shorten detection time to 1 to 2 days, although the sensitivities of different shell vial cultures are variable.

Isolated viruses can be identified as adenoviruses by various immunologic techniques; e.g., immunofluorescence, enzyme immunoassay (EIA), PCR, or latex agglutination. Serotyping of the isolates can be done by neutralization, or with some isolates, by hemagglutination inhibition. Reference antisera are available from American Type Culture Collection. Today, serotyping has been largely replaced by genotyping using PCR combined with sequencing, probe hybridization or restriction endonuclease analysis (62,228–230). Whole genome sequencing is needed for in-depth analysis of co-evolution versus intertypic recombination events for the designation of novel types (65, 70, 231, 232). Next generation sequencing will facilitate these efforts (233).

Antigen Detection

Monoclonal or polyclonal antibodies directed against the group-specific hexon antigen can be used for the direct detection of adenoviruses in clinical specimens. Commercial monoclonal antibodies and immunoassays, showing similar performances as laboratory-designed tests, are available.

In respiratory and eye specimens, infected cells can be detected by immunofluorescence, whereas immunoassay methods (EIA and time-resolved fluoroimmunoassay) can be used to detect both cell-bound and free antigenic proteins. Nasopharyngeal aspirates are preferable respiratory specimens for immunoassays, but for adenovirus, throat swabs also have good performance (234). The best sensitivity is obtained if the specimen is collected during the first 4 to 5 days after onset of illness, but some patients remain positive for 2 to 3 weeks. The sensitivity of the immunoassay methods has been 75 to 90% for children, compared to

culture or serology, but is considerably lower for adults (144,235–238). Compared to culture, immunofluorescence has a lower sensitivity than immunoassays (239). There are several commercial point-of-care tests for adenovirus. A meta-analysis of Japanese studies on immunochromatography tests reported 88%, 91%, and 67% sensitivities and 97%, 98%, and 100% specificities for throat, stool, and conjunctival specimens, respectively (240). A rapid test of adenovirus conjunctivitis (AdenoPlus), designed for direct sampling of tear fluid with a sterile collector and automatic transfer of the sample to the test strip, showed 85% sensitivity and 98% specificity as compared to PCR (241). EIAs have also been used to detect adenovirus antigens in conjunctival specimens. Compared to culture, EIA has shown a sensitivity of 70 to 80%, when specimens were collected early in the course of illness.

In fecal specimens, adenovirus antigens can be detected by immunoassays and by latex agglutination. Antigen detection is especially suitable because the enteric types 40 and 41 grow poorly in cell culture and stool samples are often cytotoxic. In addition to group-specific immunoassays, immunoassays specific for the enteric types have also been developed. The sensitivity of EIAs for enteric adenoviruses in stool has been 85 to 100%, compared to culture (144, 242). Direct visualization by EM is also used to detect adenoviruses in stool, although it is impractical under most diagnostic circumstances. The characteristic morphology of adenoviruses makes them easy to identify by EM and allows a rapid diagnosis whenever the amount of viral particles in the specimen is large enough. Enteric adenoviruses occur in stools in considerably larger quantities than respiratory adenoviruses, and the sensitivity of EM is comparable to that of immunoassays. The enteric adenoviruses can be identified by the use of immuno-EM.

PCR Tests

Adenovirus DNA can be detected directly from respiratory specimens, plasma, conjunctivae, stools, urine, and genital specimens using PCR. As with many other respiratory viruses, PCR is more sensitive than conventional virus culture or virus antigen detection. Specificity of PCR can be confirmed through sequencing. In one study of 1,038 samples from children with respiratory illness, 130 specimens were positive for adenovirus by PCR compared to 29 by FA testing (243). In another study of 181 respiratory samples from children, virus culture and direct immunofluorescence identified 7 positive samples, compared to 17 by real-time PCR (244). Similar test sensitivity has been obtained for nasopharyngeal aspirates and throat swabs (234). The high number of types and continuous evolution of new variants are challenges for PCR test development. The first priority in clinical setting is adenovirus identification; the type may become relevant later. Therefore, the conserved motifs of the hexon gene have been the target for quantitative pan-adenovirus PCRs, and protocols have been developed that use 1 to 6 different primer and probe sets (245). Multiplex PCR methods, including adenovirus as one of the targets, have been developed for the detection of respiratory, enteric, and ocular spectrum of infections (246–248). Multiplex PCR has lower sensitivity compared to individual PCRs, and adenovirus might be a particularly difficult target for these assays (249). If a severe adenovirus infection is suspected on clinical grounds, a negative multiplex PCR result should be confirmed with a more sensitive singleplex test.

Quantitative detection of adenovirus is helpful for clinical decisions (250). Since PCR may detect latent adenovirus in the respiratory, gastrointestinal, or urinary tract, a high adenovirus DNA load may be more often associated with active disease or more severe disease (226,251–253). *In situ* hybridization is a valuable method in pathogenesis studies, but it is rarely applicable in everyday diagnosis (254).

Serology

Serological studies of acute and convalescent sera may be needed to establish evidence of infection. Significant increases in titer of antibody can be measured to the common hexon antigen by complement fixation or by EIA. The sensitivity of complement fixation is about 50 to 70% and that of EIA is about 70 to 80%, compared to virus antigen detection (144). In small children, seroresponses are more attenuated than in older children and adults, and immunocompromised hosts may fail to mount responses. IgM antibodies are detectable in 20 to 50% of infections, but the immune responses are often poor and difficult to interpret. Neutralization and hemagglutination inhibition tests are sensitive but measure type-specific antibodies, so these assays are not suitable for routine diagnosis.

PREVENTION

Isolation of patients with adenoviral illness has not been recommended routinely. Detection of adenoviruses by PCR in the air and on surfaces (81, 82) suggest that proper cleaning, isolation of patients with suspected or confirmed cases of adenovirus infection, and restriction of new admissions may be essential in limiting the risk of nosocomial spread. As droplet precautions may not be effective, airborne precautions with a properly ventilated isolation room should be considered when the patient is treated in the intensive care unit and for patients with severe adenovirus type 3, 7, or 14 infections.

Rigorous hand-washing both before and after contact with the patient is recommended, but routine soap and water may not reliably remove adenovirus. An 85% ethanol hand gel reduced significantly the infectivity titer of adenovirus in 2 minutes (255). However, adenoviruses are resistant to many disinfectants, and the use of 70% isopropyl alcohol in disinfection of instruments, for example, is not reliable (88). The antiadenoviral activity of chlorhexidine is low. Surfaces should be treated with 85 to 95% alcohol for at least 2 minutes or with sodium hypochlorite for 10 minutes (5). Use of disposable gloves should be considered when examining a patient with suspected adenovirus infection. During epidemics, medical staff should be instructed not to rub their eyes or to do so only with a clean tissue or paper towel (192).

Hospital outbreaks have been controlled by grouping of patients into cohorts; the use of gloves, gowns, and goggles; and the exclusion of symptomatic staff from the unit. Environmental surfaces have been disinfected daily with sodium hypochlorite. Dirty towels may be the source of infection in outbreaks, and disposable paper towels or hot-air blowers have been recommended. Table 3 lists preventive measures for epidemic keratoconjunctivitis.

Immunoprophylaxis

Based on the observations that adenovirus infects the gastrointestinal tract but uncommonly causes gastrointestinal illness in adults, a safe and effective live oral adenovirus vaccine containing wild types 4 and 7 in enteric tablets was developed (256). These adenovirus strains replicate in the intestine and induce type-specific neutralizing serum antibodies. Since 1971, the vaccine program dramatically

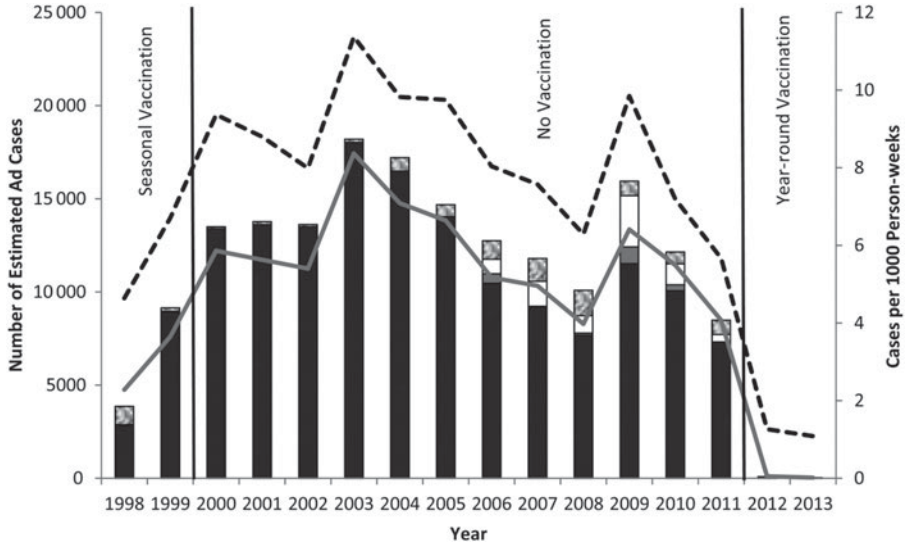

FIGURE 6 Use of adenovirus vaccines and febrile respiratory illness and adenovirus infection rates among United States Army trainees from 1998 to 2013. Reprinted from reference (8) with permission.

reduced adenoviral disease rates by 95 to 99% and total respiratory disease rates by 50 to 60% in the U.S. military, findings demonstrating the dominant role of adenovirus infections in military populations (Fig. 6). Due to the cessation of production by the sole manufacturer, no adenovirus vaccine was available from 1999 to 2012. During that time a dramatic increase in the occurrence of adenovirus infections was recorded. Two years after reintroduction of the vaccine, a 100-fold decline in adenovirus disease burden was reported. It is estimated that adenovirus vaccines now prevent approximately 13,000 febrile illnesses per year saving approximately 50 million dollars per year (8).

TREATMENT

At present there is no specific antiviral treatment of proven value for adenovirus infections. Ribavirin, ganciclovir, valganciclovir, cidofovir, and brincidofovir (formerly CMX-001) are variably active against adenoviruses *in vitro* and thus potentially effective for treatment. Ribavirin shows *in vitro* inhibitory activity only for group C adenoviruses, and intravenous ribavirin has poor or no efficacy in the treatment of severe adenoviral disease (5). A great number of case-series studies have provided anecdotal support for the clinical efficacy of cidofovir (a monophosphate nucleotide analog of cytosine) treatment, especially when given early and combined with withdrawal of immunosuppression (215, 257). Cidofovir is commonly used in the clinic as preemptive or as therapeutic treatment, but its use is associated with high rates of nephrotoxicity, and, in case of severe adenovirus disease, the efficacy of cidofovir is limited. However, prospective randomized clinical trials have not been carried out. In many centers, adenovirus DNA loads in plasma are monitored weekly in HSCT patients, and preemptive cidofovir treatment is started when adenovirus DNA is detected or increases progressively. Cidofovir treatment led to stable or reduced viral load in 70% of 18 HSCT children with blood adenovirus load greater than 1×10^3 copies per milliliter (214). Among 135 patients, mostly HSCT recipients, who had been treated with cidofovir (5 mg/kg of body weight), once per week for 1 week to 11 months, renal toxicity (mostly mild proteinuria or mild elevation of the serum creatinine) was recorded in 13%. Of 20 patients with pneumonia, 9 (45%) survived. Among patients treated with 1 milligram per kilogram, three times per week, for 2 weeks to 8 months, renal toxicity developed in 7 (29%) of 24 patients, but all 6 patients with pneumonia survived (258). A decrease in the plasma virus load has been shown to predict a clinical response. On the other hand, retrospective studies have shown that antiviral therapy may not be necessary for all children who develop adenovirus viremia after BMT (215). In neonates with disseminated infection, cidofovir or ribavirin treatment did not improve outcomes (208).

Brincidofovir is a promising orally bioavailable lipid conjugate of cidofovir. It is not associated with nephrotoxicity or myelosuppression but causes diarrhea as a dose-limiting side effect. It is *in vitro* 65-fold more potent against adenoviruses than cidofovir. A controlled clinical trial to evaluate the safety and efficacy of brincidofovir for adenovirus disease in 200 HCT recipients have been recently completed (5, 259, 260).

Proper T cell function is crucial for clearance of adenoviral infection. Adoptive transfer of adenovirus-specific T cells is a promising treatment for allo-HSCT recipients not responding to antiviral chemotherapy (5). Peripheral blood mononuclear cells of the HSCT donor are stimulated *in vitro* with adenovirus hexon protein and enriched and transferred to the patient. With this treatment protocol adenovirus-related mortality was 9.5% in 21 patients who had immunological response to the therapy, compared with 100% mortality in 8 patients did not respond (261). Hemorrhagic cystitis after bone marrow and renal transplantation is treated with steroid pulse therapy, but the value of this therapy is unproven. Intravesical instillation of cidofovir in the treatment of adenovirus-induced hemorrhagic cystitis has been reported (205).

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Polyomaviruses

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Polyomavirus infections are widespread among humans and animals. The prototype of this viral family, polyoma virus of mice, was discovered in 1953 as an agent capable of producing tumors in its natural host (1). A second polyomavirus, murine K virus (KV; now known as mouse pneumotropic virus or MPtV), was discovered in 1952 (2). The simian polyomavirus, SV40, was discovered in 1960 as a contaminant of lots of rhesus monkey kidney cells used to prepare polio vaccine stocks (3). Infectious SV40, subsequently detected in both the Salk and Sabin polio vaccines, as well as in adenovirus vaccines, was inadvertently administered to millions of individuals worldwide (3).

The first evidence that polyomaviruses might also be infectious for humans came in 1965, when Zu Rhein et al. and Silverman and Rubinstein independently reported the electron microscopic detection of structures resembling polyomaviruses in brain sections from patients with the fatal demyelinating disease, progressive multifocal leukoencephalopathy (PML) (4). Isolation of the agent was achieved in 1971, when Padgett et al. recovered a previously unknown human polyomavirus, JC virus (JCPyV), by inoculation of PML brain material from patient J.C. into primary cultures of human fetal glial cells (5). In that same year, a second polyomavirus, BK virus (BKPyV) was recovered from the urine of a human renal transplant patient (6). BKPyV has since been repeatedly recovered from urine and also been associated with hemorrhagic cystitis in allogeneic hematopoietic stem cell transplant patients (7) and nephropathy/interstitial nephritis in renal transplant patients.

For over 40 years, JCPyV and BKPyV were the only polyomaviruses unequivocally associated with human infections. Recently, however, 11 additional human polyomaviruses have been identified (Table 1), and nearly 100 additional polyomaviruses have been recovered from nonhuman primates, other mammals, birds, and fish (8, 9). An unambiguous link to tissue pathology and disease is missing for most polyomaviruses. This chapter will emphasize the major polyomaviruses that have been associated with human clinical illness and cancer: JCPyV, BKPyV, MCPyV, and TSPyV. The simian *betapolyomavirus* SV40, however, remains controversial as a cause of human illness and neoplasia (10).

VIROLOGY

Classification

Polyomaviruses were initially considered a genus within the family *Papovaviridae*, which included papillomaviruses and polyomaviruses. In 2000, these agents were reclassified as two separate families, *Papillomaviridae* and *Polyomaviridae*. Subsequently, the rapidly increasing number of polyomaviruses isolated from human and nonhuman animal species led the *Polyomaviridae* Study Group of the International Committee on Taxonomy of Viruses (ICTV) to propose dividing the polyomaviruses into three separate genera: *Orthopolyomavirus*, which included JCPyV, BKPyV, and SV40; *Wukipolyomavirus*; and *Avipolyomavirus*, which was restricted to polyomaviruses infecting birds. As of December 2015, a new classification system has been proposed, based on the observed distance between large T coding sequences: this designates four genera: *Alpha-*, *Beta-*, *Gamma-*, and *Deltapolyomavirus* according to host species and order of detection (9). The recently recognized human polyomaviruses are distributed across *Alphapolyomaviridae* HPyV8, HPyV9, HPyV12, and NJPyV; the *Betapolyomaviridae* KIPyV and WUPyV; and the *Deltapolyomaviridae* HPyV6, HPyV7, and HPyV11 genera (9). This classification has been accepted by the ICTV but is still subject to modification.

Virus Structure and Composition

Polyomaviruses are unenveloped 40 to 45 nm, icosahedral agents encapsidating a supercoiled, circular, double-stranded DNA genome wrapped around two histones (Figure 1). The genome consists of 4,900 to 5,900 base pairs (bp), which comprise three functional regions (Figure 2). The noncoding control region (NCCR) of approximately 400 bp harbors the origin of replication (ori), and promoter/enhancer units directing the bidirectional expression of the early and late viral genes in concert with host cell factors reflecting differentiation and activation. The early viral gene region (EVGR) of approximately 2,500 bp encodes the key regulatory proteins including large T-antigen (LTag) and the small T-antigen (sTag). The primary EVGR transcript is also spliced to give rise to several smaller T-Ag derivatives with partly overlapping domains, which are replication cycle, host cell, or species dependent. Examples include LTag and truncT' of JCPyV and MCPyV, respectively, or the middle T-antigen (mTag) of

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TABLE 1 Human polyomaviruses[1]

Common agent name (abbreviation)	Taxonomic name	Major site(s) of persistence	Infection in immunologically normal individuals	Symptomatic infection, immunocompromised individuals
Genus *Alphapolyomavirus*				
Merkel cell polyomavirus MCPyV1)	**Human polyomavirus 5**	**Skin**	**Unknown**	**Isolated from 80% of cutaneous Merkel cell carcinomas**
Trichodysplasia spinulosa (TSPyV)	Human polyomavirus 8	**Skin**	**Unknown**	**Trichodysplasia Spinulosa**
Human polyomavirus 9 (HPyV9)	Human polyomavirus 9	Unknown: recovered from plasma and urine	Unknown	Unknown
Human polyomavirus 12 (HPyV12)	Human polyomavirus 12	Liver (colon, rectum)	Unknown	Unknown
New Jersey polyomavirus (NJPyV)	**Human polyomavirus 13**	**Unknown**	**Unknown**	**Microvasculitis of muscle, necrotizing dermopathy, and retinal blindness in a pancreatic transplant patient (1 case)**
Genus *Betapolyomavirus*				
BK virus (BK polyomavirus) (BKPyV, BKPyV)	**Human polyomavirus 1**	**Kidney**	**Inapparent or mild Periodic viruria**	**Nephropathy Hemorrhagic cystitis Ureteral obstruction**
JC virus (JC polyomavirus) (JCPyV, JCPyV)	**Human polyomavirus 2**	**Kidney (brain, PBMCs, possibly other organs)**	**Inapparent or mild Periodic viruria**	**PML (cerebellar granule cell infection) (cortical encephalitis)**
KI virus (KI polyomavirus) (KI PyV)	Human polyomavirus 3	Respiratory tract, possibly gastrointestinal tract	Possible but unconfirmed association with respiratory tract infection	Possible but unconfirmed association with respiratory tract infection
WU virus (WU polyomavirus) WUPyV	Human polyomavirus 4	Respiratory tract, possibly gastrointestinal tract	Possible but unconfirmed association with respiratory tract infections	Possible but unconfirmed association with respiratory tract infections
Genus *Deltapolyomavirus*				
Human polyomavirus 6 (HPyV6)	Human polyomavirus 6	Skin	Unknown	Unknown
Human polyomavirus 7 (HPyV7)	Human polyomavirus 7	Skin	Unknown	Unknown
MW polyomavirus (MWPyV)	Human polyomavirus 10	Gastrointestinal tract	Unknown	Unknown
STL polyomavirus (STLPyV)	Human polyomavirus 11	Tonsillar tissue (gastrointestinal tract?)	Chronic tonsillitis	Unknown

[1]Agents associated with disease are shown in bold type.

mouse polyomavirus (MPyV) and the human MCPyV (11). The late viral gene region (LVGR) of approximately 2,900 bp encodes the capsid proteins Vp1, Vp2, and Vp3, which are posttranscriptionally derived from different splice and translation start signals. In the 5′ end of the LVGR, some polyomavirus species including JCPyV, BKPyV, and SV40 encode a small regulatory leader protein called agnoprotein. In addition, microRNAs have been detected in the distal LVGR; these are complementary to EVGR transcripts and may play a role in down-regulation of T-antigen expression (11).

JCPyV, BKPyV, and SV40 DNAs exhibit extensive (69% to 75%) DNA homology, with greatest sequence divergence being found in the regulatory region (RR), on the late side of the ori (11). In contrast, although the more newly discovered polyomaviruses are phylogenetically related to other human and primate polyomaviruses in the early region of their genomes, each of these newer agents is divergent from JCPyV, BKPyV, and SV40 in the amino acid homology in the LVGR encoded Vp1 (12).

Although the genomes of JCPyV, BKPyV, and SV40 encode only single serotypes of virus; extensive variation

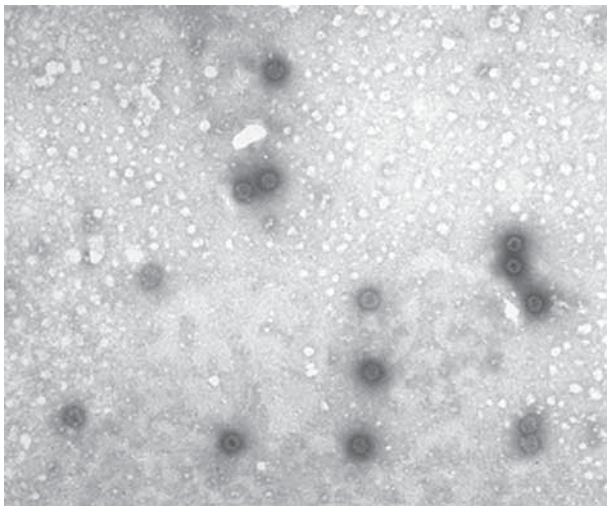

FIGURE 1 Electron micrograph of BKPyV extracted from human fetal kidney cells, concentrated by ultracentrifugation, and stained with 2% phosphotungstic acid, showing characteristic 42 nm particles.

may occur in the NCRRs of each (11), resulting in multiple genotypes (often referred to as "strains") of each agent. Genotypes that are shed in urine and involved in transmission of infection are termed "archetypal." Genotypes differing from the archetype in their rNCCR are termed "variant" or "rearranged" (13). As discussed below, variance from archetypal genomic sequences has been postulated to account for differences in the biological behavior of these agents, including host range, regulation of viral growth, and ability to cause disease. In the case of SV40, archetypal genotypes isolated directly from simians usually contain only a single 72 bp enhancer element, whereas partial duplication of this region is characteristic of genotypes of virus cultivated *in vitro* (14).

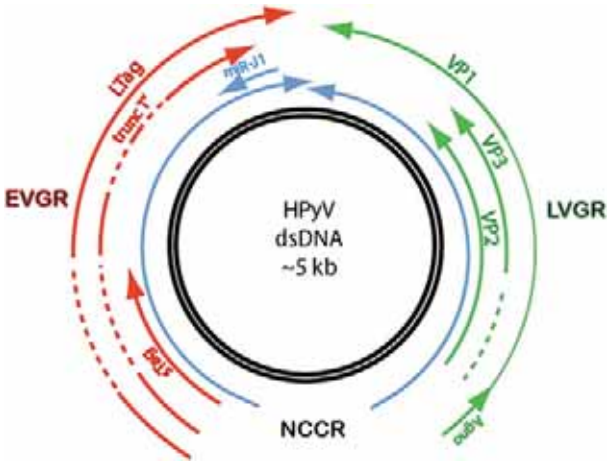

FIGURE 2 Schematic representation of a human polyomavirus genome, made up of circular double-stranded DNA. Regions comprising the NCCR are shown in blue; those comprising the EVGR are shown in red: these encode LTag, truncT' as found in MCPyV, and sTag. LVGR sequences encoding VP1, VP2, VP3, and the agno protein are shown in red. The agno protein-encoding gene is not found in every HPyV genome.

BKPyV was initially considered to be comprised of prototypic Dunlop (DUN) and five other genotypes, MM, GS, MG, AS, and RF (11, 15). Subsequently, BKPyV was divided into four major genotypes (I, II, III, and IV), based on heterogeneity of VP1 gene and immunoreactivity (15), with genotype I being most common worldwide, and subtype IV being common in Europe and East Asia but rare in Africa (16, 17). Group I comprises the DUN genotype, as well as two other genotypes, MM and GS. Group II encodes the SB genotype; group III the AS genotype; and group IV the MG and RF genotypes (11). GS and MM viruses have sequence differences around the replication origin. MGV and RFV, although antigenically similar to BKPyV, have a bipartite genome consisting of two complementing defective molecules, one having a deletion corresponding to the BKPyV early region, and the other, to the BKPyV late region (18, 19). More recently, BKPyV has been further classified into subgroups, which also appear to correlate with geographic distribution (16, 17). Individuals may harbor more than one genotype of virus.

Regulatory and Structural Proteins

Polyomaviruses encode two groups of viral proteins: "early proteins," which are regulatory and control viral synthesis but are not components of the virion; and "late" proteins, which include the structural virion proteins (Table 2) (11). EVGR and LVGR are separated at their 5′ termini by the NCCR of approximately 400 bp, which contain a single origin of replication (Figure 2). The NCCR plays an important role in controlling EVGR and LVGR expression and also in initiating viral DNA replication.

The early proteins have been most thoroughly studied in the case of SV40. The roles of these proteins in SV40 synthesis are thought to apply to other polyomaviruses as well. The most important early protein is large T-antigen (LTag), a 708 amino acid protein, which is expressed predominantly in the cell nucleus but is also present at the cell membrane (Figure 3) (11, 20). LTag binds to the viral origin of replication as well as the NCCR and has multiple functions, many of which are accomplished by the ability of LTag to bind to and modulate the activity of different host cell proteins (11). Functions of LTag include initiation and execution of viral genome replication, and regulation of synthesis of both viral and cell proteins (Table 2) (20). LTag is the only viral protein essential for viral DNA synthesis and interacts with multiple cellular proteins including tumor suppressor proteins pRb, p107, p130, and p53 (Figure 4). LTag also plays an essential role in "virus-induced" cell transformation (11, 20), such that the presence of LTag in cells has been used as a marker for polyomavirus-mediated cell transformation (Figure 4) (20). LTag also serves as a recognition site for cytotoxic T-lymphocyte-mediated immune control (21, 22). sTag is a histone-rich protein that shares its N-terminus with LTag but has a unique C-terminus. The functions of sTag and of the late, nonstructural agno protein are outlined in Table 3 (11, 23, 24, 25).

Three structural proteins comprise the viral capsid in most human polyomaviruses: the 45 kDa protein Vp1, and two smaller proteins, Vp2 and Vp3. These are arranged into 72 capsomeres, each comprised of 5 molecules of VP1 and one molecule of VP2 or VP3. VP1 is the only protein exposed on the capsomere surface and is the major protein involved in viral attachment to cells (11). In contrast, MCPyV contains only VP1 and VP2; VP3 is not detected (26). A smaller derivative called Vp4 has been reported to

TABLE 2 Functions of early and late polyomavirus-encoded proteins

Large T-antigen	
1) Viral DNA replication	T-Ag forms a double hexamer and in the presence of ATP causes untwisting of the early viral DNA palindrome within the origin. Using its helicase activity, the complex unwinds the viral DNA bidirectionally. Cellular proteins, topoisomerase I and DNA polymerase/primase are recruited to the replication origin to form a replication complex that is involved in the initiation of the replication of viral DNA molecules.
2) Activation of late viral gene expression	T-Ag effects an alteration of the cellular repressors that normally bind near the late promoter. This derepression, perhaps in combination with recruitment of transcription factors, activates late transcription. Alternatively, replicated viral DNAs appear in sufficient amounts to titrate the repressors.
3) Down regulation of T-Ag expression	Two possible mechanisms for this down-regulation have been reported: 1) T-Ag activates expression of the late genes at the late promoter by helping to recruit transcription factors. Activation of the late promoter sequesters the transcription factors also required for the activation of the T-Ag promoter causing its own down-regulation. 2) Autoregulation: T-Ag binds to viral DNA at sites that block expression of the T-Ag gene.
4) Initiation and maintenance of cell transformation	By binding to and/or inactivating tumor suppressors p53, Rb, and perhaps several others, the suppression or control of cell growth is then reversed and the cells become morphologically transformed and display several parameters of transformation. Also, the cells may form tumors in appropriate animals and can assume an immortal phenotype in culture. T-Ag may be required for immortalization of polyomavirus-transformed cells.
Small T-antigen	
1) Intracellular accumulations of viral DNA and viral replication. Regulates PP2A.	
2) Reverses the apoptotic effect of large T-Ag.	
3) Causes cell cycle progression. Increases tumorgenicity of LTag transformed cells.	
4) Binds several cellular proteins, e.g., p300, PP2A.	
5) Induces telomerase and AKT activities.	
Late (Capsid and Agno) proteins	
VP1	Major capsid protein: involved in virus attachment to cell surface receptors.
VP2, VP3	Minor capsid proteins.
Agno protein	1) May serve a role in viral morphogenesis perhaps by retarding polymerization. 2) May help with intracellular migration of VP1 to the perinuclear region and entry of VP1 into the nucleus with consequent viral assembly. 3) Involved with late transcriptional regulation. 4) May play a role in cell to cell spread of virus.

function as a viroporin for SV40 (27). Extensive antigenic similarity exists between the large and small T-Ags of JCPyV, BKPyV, and SV40. A lesser degree of antigenic cross-reactivity exists among intact JCPyV, BKPyV, and SV40 virions or among the newer polyomaviruses (28, 29, 30).

Biology

Virus Attachment and Entry into Cells

Most PyV capsids bind host cell membrane structures, which consist of glucose: galactose, N-acteyl-galactosamine, and N-acetyl-neuraminic acid, connected by glucosidic linkages to lipids in the host cell membrane and to each other. JCPyV binds to two receptors in brain: an α2,6-linked sialic acid on the lactoseries tetrasaccharide c (LSTc) glycan and also the 5-HT2AR serotonin receptor (31, 32, 33). The distribution of LSTc, serotonin receptors, and virus binding sites overlap in the kidney, a major site of JCPyV persistence and in the choroid plexus (34). Within the brain, however, the two receptors are not coexpressed: LSTc is found on microglia, vascular endothelial cells, and cells in the choroid plexus, whereas 5-HT2AR serotonin receptors are found on oligodendrocytes and astrocytes. These data suggest that LSTc might allow JCPyV binding at the blood-brain barrier, but that 5-HT2AR serotonin receptors, rather than LSTc, are necessary for JCPyV attachment to its target glial cell populations (34).

BKPyV attachment to cells involves binding of viral pentamers to gangliosides GD1b carrying one branch of β1,4-*N*-acteyl-galactosamine-β1,4-galacose and one branch of α2,3 *N*-acetyl-neuraminic acid- α2,8 *N*-acetyl-neuraminic acid (35). BKPyV also binds to GT1b, which corresponds to a GD1b carrying one additional α2,3-*N*-acetyl-neuraminic acid coupled to the β1,4-*N*-acteyl-galactosamine (35). Addition of the gangliosides GD1b and GT1b to LNCaP cells, which are normally resistant to BKPyV infection, makes the cells susceptible to infection by the virus (35). The events involved in attachment and uptake of the more recently discovered polyomavirus are less well understood, because of the difficulty in generating replication competent PyV virions from *in vitro* cell culture systems. MCPyV has been reported to bind to GT1B (36) but may also undergo a two-step attachment and entry process, with initial binding of VP1 to heparin sulfate and other sulfated glycosoamines, followed by transfer of the virus to a sialylated glycan, which appears to play a role in viral uptake (37).

The ability of polyomaviruses to bind to cell membrane glycosylation structures enables many of these agents, such as

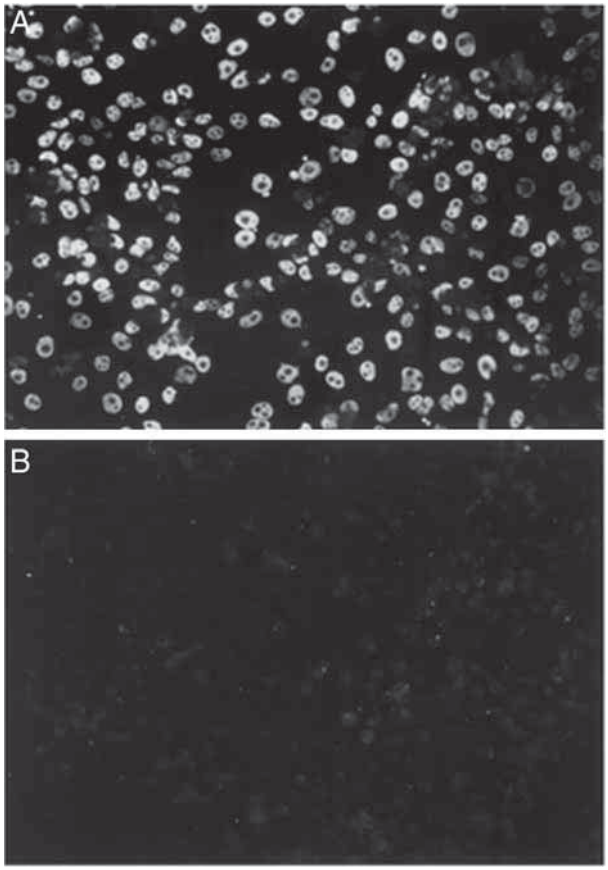

FIGURE 3 (A) Identification of SV40 T-Ag by immunofluorescence in transformed simian TC-7 kidney cells. Cells were grown in a monolayer and reacted with monoclonal antibodies PAb 416, PAb 108, and PAb 101 directed against T-Ag as described by O'Neill et al. (272). (B) Naïve TC-7 cells (not containing T-Ag-encoding DNA sequences) reacted with monoclonal antibodies as in panel A (negative control).

JCPyV and BKPyV, to agglutinate red blood cells of various species. In contrast, SV40 does not hemagglutinate as it binds to the branched glycan GM1 (β1,4-glucose-β1,4-glactose-β1,4-*N*-acteyl-galactosamine and one α2,3-*N*-acetyl-neuraminic acid), which is not found on erythrocytes.

BKPyV and SV40 enter cells by endocytosis into caveosomes and from there into the endoplasmic reticulum (38, 39). Virus-containing vesicles are then transported intracellularly to fuse with the outer membrane of the cell nucleus, releasing viral particles into perinuclear cisternae. Uncoating of SV40 and MCPyV DNAs begins in the endoplasmic reticulum (11). In contrast, internalization of JCPyV involves endocytosis of the virus within clathrin-coated pits, and transport of JCPyV to the cell nucleus involves microtubules and microfilaments (11). Uncoating of JCPyV DNA is thought to occur exclusively within the cell nucleus.

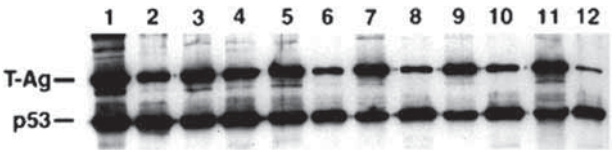

FIGURE 4 Western blot of SV40 T-Ag and p53 from transformed human cells. SV40 T-Ag was immunoprecipitated in even-numbered lanes and p53 was immunoprecipitated in odd-numbered lanes, and the immunoprecipitates were subjected to electrophoresis in agarose gels and analyzed by Western blotting using a cocktail of antibodies to T-Ag or p53 (95). The illustration shows that immunoprecipitation of either protein results in coprecipitation of the second protein.

Viral Replication

The steps of viral replication have been most clearly defined for SV40 and BKPyV. Other polyomaviruses are thought to replicate in a similar fashion. Replication of the SV40 genome occurs in two stages, with early viral genes being transcribed off of one DNA strand in one direction and late viral genes being transcribed after the early genes and off the other strand in the opposite direction (11). Polyomavirus gene transcription is governed by *cis*-acting sequences in the regulatory region (11). The early promoter of SV40 is located near the origin of replication and contains a TATA box, three G-C rich regions, and an enhancer region of 72 bp tandem repeats all contained within the NCCR (11). The early G-C rich regions are bound by the Sp1 cellular transcription factor and the enhancer region, which are thought to interact with other transcription factors. EVGR and LVGR expression is critically controlled by a hierarchy of transcription factors, in which Sp1 and Ets1 sites play a major role (40). These sites are altered in natural BKPyV NCCR variants from immunocompromised patients with nephropathy (41), hemorrhagic cystitis (42), and disseminated infection (43, 40). Transcription is mediated by cellular RNA polymerase II, resulting first in the production of EVGR mRNAs encoding LTag and sTag (11). During late transcription there is differential splicing to form the mRNAs encoding the agnoprotein, as well as VP1, VP2, and VP3. Assembly of virions occurs in the cell nucleus, followed by release of viral particles during cell lysis (11).

Host Range

Polyomaviruses are extremely species-specific and are for the most part unable to cause productive infection in unrelated species. SV40 represents an exception to this rule: the virus naturally infects rhesus and cynomolgus monkeys but was shown to produce limited infection in humans following exposure to contaminated vaccines (44). Similarly, BKPyV has been shown in one study to cause systemic infection and interstitial nephritis in immunosuppressed cynomolgus monkeys (45). Species specificity and ability to infect specific cell populations are controlled by regulatory elements unique to each virus and their interaction with host cell proteins (11). Inoculation of laboratory rodents (or in the case of JCPyV or BKPyV, nonhuman primates) with JCPyV, BKPyV, or SV40 causes a variety of neoplasms, usually at or near the site of inoculation. Other animal models are discussed in the Pathogenesis section.

Growth in Cell Culture

SV40, BKPyV, and PML-associated genotypes of JCPyV can be grown in primary human fetal glial (PHFG) cells. In this culture system, SV40 results in a progressive lytic infection of certain cell populations, resulting in high titers of infectious virus, and a transforming infection in others (Figure 5) (14, 46). Inoculation of PHFG cultures with BKPyV produces an infection that is nearly identical to that seen with

TABLE 3 Major immunotherapeutic and cytoxic agents associated with PML

Agent	Mechanism of action	Therapeutic use	Frequency of PML	Duration of treatment before diagnosis	Treatment	Mortality
Natalizumab	Binds to *a*4 subunit of *a*4*β*1 and *a*4*β*7 integrins expressed on leukocytes. Inhibits lymphocyte trafficking into brain	Multiple sclerosis Crohn's disease	8.5/1,000 in JCPyV-positive patients treated for 49–72 months; 13/1,000 in patients with prior immunosuppression	Months to years	Discontinue drug. Plasma exchange plus immunoadsorption	23%[1]
Rituximab	Anti-CD20 monoclonal. Produces destruction of B lymphocytes and pre-B lymphocytes	Lymphoproliferative disorders; collage-vascular diseases and other autoimmune disorders; AIDS-related lymphomas	1:30,000 (most cases in patients receiving multiple immunosuppressive drugs	Months to years	Discontinue drug. Plasma exchange. Use of immunoadsorption not reported	Death in most reported cases[2]
Alemtuzumab	Binds to CD52 receptors on lymphocytes	B-cell chronic lymphocytic lymphoma Multiple sclerosis	Rare cases in B-CLL, none as yet in multiple sclerosis	Months	Discontinue drug. Use of plasma exchange or immunoadsorption not reported	Unknown[3]
Brentuximab vedotin	Binds to CD30 receptors on Reed-Sternberg and other Hodgkins or leukemia cells, activated T cells, resting B cells	Anaplastic large cell and Hodgkin lymphomas	Unknown	Weeks to months	Discontinue drug. Use of plasma exchange or immunoadsorption not reported	80%
Mycophenolate mofetil	Inhibits inosine monophosphate dehydrogenase, involved in B- and T-cell proliferation	Solid organ transplants; systemic lupus erythematosus; other autoimmune diseases Hodgkin's disease	Rare	Weeks to months	Discontinue drug.	Death in most reported cases[2]
Fludarabine	Inhibits DNA polymerase alpha, DNA primase, ribonucleotide reductase	Chronic lymphocytic leukemia	0.52% of treated patients	Months	Unknown	Death in most reported cases
Dimethyl Fumarate	Mechanism unknown	Multiple sclerosis Psoriasis	< 10 cases reported	Months	Discontinue drug	Unknown[3]
Fingolimod	Prevents transit of lymphocytes out of lymphoid organs	Multiple sclerosis	< 10 cases reported	Months[4]	Discontinue drug	Unknown[3]

[1]As of March 2015.
[2]Most reported cases are in individuals treated with two or more immunosuppressive agents.
[3]Numbers of reported cases too small to allow estimation of overall mortality.
[4]Some cases have occurred in patients following transition from natalizumab to fingolimod.

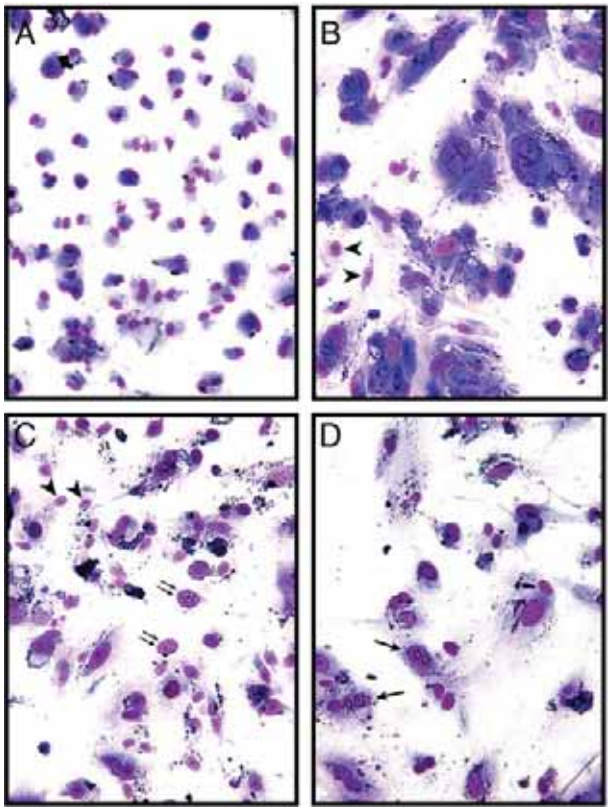

FIGURE 5 Comparative cytopathic effects produced by JCPyV, BKPyV, and SV40 in primary cultures of human fetal brain cells. Cultures were infected with a 1/10 dilution of the PML-associated Mad-1 genotype of JCPyV or with 1/1,000 dilutions of SV40 or BKPyV. (A) Uninfected human fetal brain cells containing relatively small, moderately stained nuclei. (B) Cultures infected with a 1/10 dilution of the PML-associated Mad-1 strain of JCPyV, showing cells with enlarged nuclei and multinucleated cells. Occasional giant multinucleated cells were present in some cultures (not shown). (C) Cultures infected with a 1/1,000 dilution of SV40. Cultures show large, darkly staining nuclei. Some nuclei were reticulated or mottled (double arrows), but multinucleated cells were rare. Arrowheads in panels B and C designate cells with small nuclei, similar to those seen in uninfected cultures. (D) Cultures infected with a 1/1,000 dilution of BKPyV, showing enlarged, intensely stained nuclei. Some nuclei contained what appeared to be doughnut-shaped nucleoli (arrows). This was unique to BKPyV infection. As in SV40-infected cultures, multinucleated cells were rarely present. In cultures infected with BKPyV or SV40, cytopathic effect became extensive within 8 to 10 days. In cells infected with Mad-1 JCPyV, cytopathic effect did not appear until 3 to 4 weeks. (Wright's stain; magnification, ×152).

SV40, and serial passage of BKPyV-infected brain cultures results in the development of both persistently infected and transformed cell lines (Figure 5) (46). In contrast, PML-associated genotypes of JCPyV cause a productive infection in PHFG that evolves over weeks. JCPyV infection in this culture system has a more limited cytopathic effect than does that produced by BKPyV or SV40 and in some studies has been described to involve spongioblasts or oligodendroblasts almost exclusively (Figure 5) (46).

Other than their ability to replicate in PHFG cells, the culture requirements of these three agents differ considerably (47). SV40 can be isolated in cell lines derived from African green monkey kidney cells (BSC-1, CV-1, and Vero cells), and has also been propagated in several human tumor cell lines (47, 48). SV40 grows poorly in human fibroblasts, such as WI-38, and even less efficiently in HEK cells (47, 48, 49, 50). BKPyV readily replicates in primary human proximal tubular epithelial cells, which represent the natural target in nephropathy, and has become the preferred experimental model in tissue culture (51, 52, 40). BKPyV also grows in human fibroblasts, early passage primary cultures of human embryonic kidney (HEK) and PHFG cells, simian BSC-1 cells, and, to a limited extent, in simian Vero cells (53). JCPyV was initially isolated from PML brains using PHFG cells, and this culture system has been used most extensively in studies of the virus (5). Although several other cell culture systems have been developed, these have been less extensively employed (4). The human glial cell–derived line SVGp12 expressing SV40 LTag supports JCPyV replication, but because of its contamination with BKPyV, researchers have resorted to the BKPyV-free clone SVG-A (54). Limited growth of the JCPyV archetype has been shown to occur in PHFG cells, and very slow growth of the virus also occurs in HEK and human endothelial cultures (55). Efficient propagation of the archetype forms of both BKPyV and JCPyV has been reported in 293TT (human embryonic kidney) cells overexpressing SV40 T-Ag (56). Efficient propagation of the archetype forms of both BKPyV and JCPyV has been reported in cells expressing the SV40 LTag such as HEK293TT, COS-7, SVG-A cells, and SVGp12, which carries infectious BKPyV (56, 54). Although transduction of cells in culture has been reported with MCPyV DNA (57), infection of cells in culture using the newer polyomaviruses has not yet been reported.

Cell Transformation by Polyomaviruses

In primary cell cultures or cell lines that are "permissive," polyomaviruses replicate to generate progeny virus. Infection of cells that are "nonpermissive" for these viruses, however, is characterized by integration of the viral genome into that of its host; these cells usually express early but not late viral proteins, develop distinctive biological and biochemical properties such as the ability to grow from single cells or to grow in agar or serum-free media, and are considered transformed (11, 58). Some, but not all, transformants become capable of indefinite serial passage and are considered immortal in culture (59). In general, cell transformation has required the use of cell culture systems derived from species other than those infected in nature by the virus. Most cell transformation studies involving SV40, BKPyV, and JCPyV have employed cultured rodent cells. In addition, BKPyV and SV40 have been shown to transform primary cultures of human diploid cells, including PHFG cells, HEK cells, and fibroblasts (47). Studies of chimeric SV40/BKPyV and SV40/JCPyV LTags demonstrated that three regions of SV40 LTag are required for immortalization of human cells: the N-terminus, a central region containing the Rb-binding domain, and the C-terminus (60). The mechanisms by which immortalization occurs are incompletely understood. In the case of SV40 these appear to involve inactivation of the cellular growth suppressors pRB, p53, and SEN6 (59). Neither JCPyV nor BKPyV matches SV40 in its ability to immortalize human cells. Cell transformation by inoculation with intact MCPyV virions has not been described, but MCPyV sTag is sufficient to transform rodent fibroblast cell lines (61).

Inactivation by Chemical and Physical Agents

Polyomaviruses withstand prolonged drying at room temperature and are relatively resistant to heat. Because they lack a lipid envelope, they are unaffected by ether and other lipid solvents. The viruses can be inactivated β-propiolactone, proteolytic digestion, formaldehyde, prolonged heating to above 50°C, and exposure to ultraviolet light. The ability of these agents to persist at room temperature can present significant risks of inadvertent contamination in laboratories in which these agents have been studied.

EPIDEMIOLOGY

Polyomavirus infections are worldwide in distribution among human populations. The prevalence of antibody to JCPyV is 10% in children 5 years of age and rises to over 50% by late adult life (62, 63, 64). Serological evidence of infection by BKPyV is present in 37% of individuals by 5 years of age and 83% by adolescence, falling to 53% in individuals over 50 years of age (65, 63, 64). Prevalence of antibodies to MCPyV is over 45% by age 10 and over 80% by age 69 (66, 67), whereas seropositivity rates for KIPyV, WUPyV, and HPyV7 in adults over 21 years of age have been reported to be 55%, 69%, and 56%, respectively (68, 67). Seroprevalence of antibodies to TSPyV are 41% in children under 10 years of age rising to 70% in adults overall, but falling in older individuals (69). Antibodies to BKPyV and JCPyV have been detected in populations so isolated as to be without evidence of contact with measles and influenza viruses (70). The high prevalence of serum antibody response to JCPyV or BKPyV is not further increased in immunosuppressed patients, including those with HIV infection.

SV40 as a Human Agent

Over 150 million individuals worldwide received SV40-contaminated polio or adenovirus vaccines (3). The frequency of antibody response to SV40 in groups receiving contaminated polio vaccines was as great as 24% (71). Children receiving contaminated vaccines developed subclinical SV40 infection with prolonged fecal excretion of the virus (4), and experimentally infected volunteers showed both transient viremia and prolonged viruria (71). Up to 27% of personnel working for monkey export companies have anti-SV40 antibodies, and serological evidence of SV40 infection has been reported in 51% to 55% of laboratory workers in contact with monkeys or involved in preparation of monkey cells for tissue culture purposes (71). These data all indicate that SV40 is capable of replication in human hosts.

Serological, virological, and polymerase chain reaction (PCR) studies from a number of laboratories have also suggested human infection with SV40 or an SV40-like agent in individuals not exposed to contaminated vaccines (4, 72, 73, 74). The evidence for a human SV40-like agent has been controversial, however, in part because antibodies to SV40 in human sera have for the most part been at low titer, and because many of the reports failed to exclude cross-reacting antibodies to JCPyV or BKPyV (75, 76, 77). One serological study of sera from 699 patients, with and without cancer, detected antibodies to JCPyV or BKPyV in all patients and control subjects (75). Although antibodies reactive with SV40 were found in 6.6% of serum samples, preabsorption experiments confirmed that these were actually antibodies to JCPyV or BKPyV, which were cross-reactive with SV40. Attempts to amplify SV40 from urine of AIDS patients or from human sewage collected from widely dispersed areas in Europe and Africa were unsuccessful despite the fact that JCPyV and BKPyV were readily detected in urine and stool (78, 79). In one study, SV40 and BKPyV coinfection was reported in some patients (80), but this was not the case in molecular studies of renal tissue from 19 patients with interstitial nephritis, although these studies did identify JCPyV and BKPyV (81). Furthermore, although infectious JCPyV and BKPyV virions have been repeatedly recovered from human urine, these studies have not detected SV40, nor was SV40 detected in molecular studies of renal tissue from 19 patients with interstitial nephritis, although these studies did identify JCPyV and BKPyV (81). The existence of an SV40-like agent of humans thus remains disputed (10).

Transmission

Because no human polyomavirus has as yet been consistently associated with symptomatic illness during primary infection, the routes by which human polyomavirus infections are acquired naturally have not been defined, nor do we know the cells and tissues that support viral replication during primary infection. The most thorough data concerning polyomavirus transmission come from studies of nonhuman agents. Mouse polyomavirus is transmitted by respiratory spread (82). Mouse pneumotropic virus (MPtV) is acquired orally, with initial replication of the virus in intestinal endothelial cells (83). SV40 produced asymptomatic infection in volunteers after either oral or respiratory inoculation (71). JCPyV and BKPyV DNAs have been detected in human feces, suggesting that both viruses, like MPtV, may be transmitted by the oral route (84, 85, 86). Both JCPyV and BKPyV DNAs have been detected in human tonsils. However, PCR analysis of nasopharyngeal aspirates from children requiring hospitalization for acute respiratory illnesses detected BKPyV DNA—but not infectious virus—in only 2 of 201 patients and did not detect JCPyV DNA (87). Neither JCPyV DNA nor BKPyV DNA were identified in saliva from 60 HIV-infected adults or 10 healthy adult controls (87). However, BKPyV DNA can be detected in saliva by PCR, and the virus can productively infect submandibular and parotid gland cells lines (88). These data, in aggregate, suggest that JCPyV and BKPyV may persist in tonsillar or salivary tissues and that, in the case of BKPyV, salivary tissues might play a role in viral transmission. For the newer polyomaviruses associated with cutaneous shedding of virus, like MCPyV, infection is thought to be acquired by direct skin-to-skin contact among individuals or from environmental surfaces contaminated with virus (89). Suspected, but unproven, sites for primary infection by the newer human polyomaviruses include nasopharynx and lung for WUPyV and KIPyV; the skin for MCPyV, HPyV6, HPyV7, TSPyV, and HPyV9; and the gastrointestinal tract for MWPyV and STLPyV (90). Sexual transmission of human polyomavirus infection has not been documented, nor has detection of polyomavirus DNAs in human semen been reported.

Individuals with impaired host immunity do not appear at greater risk of acquiring polyomavirus infection. However, as will be discussed below, urinary excretion of both JCPyV and BKPyV are increased under conditions of immunosuppression, and clinical disease caused by JCPyV, BKPyV, MCPyV, and TSPyV occurs essentially only in immunosuppressed patients (91, 92).

Progressive Multifocal Leukoencephalopathy

PML is an opportunistic demyelinating infection caused by JCPyV. The disease was initially identified as an extremely

rare, almost invariably fatal condition seen in patients immunosuppressed because of hematological malignancies, cancer chemotherapy, immunosuppression for organ transplantation, or collagen vascular disease (4). The disease was also reported, but rarely, in patients with protracted granulomatous disorders (including tuberculosis or sarcoidosis), with congenital immune deficiencies, or with celiac disease (4). With the advent of the AIDS epidemic, PML became much more common, causing death in 4% of patients (93). Following development of combination antiretroviral therapy (cART), however, this number has fallen significantly, and many treated patients with AIDS-PML now survive (94, 95).

In recent years, increasing numbers of cases of PML have been reported in non-AIDS patients receiving potent monoclonal immunomodulatory agents (Table 3), as well as in individuals receiving aggressive immunosuppression for organ transplantation (96, 97, 98). The agent most commonly associated with PML has been the humanized monoclonal antibody, natalizumab (99). Natalizumab reacts with the *a*4 subunit of *a*4*β*1 and *a*4*β*7 integrins, which are expressed on the surface of all leukocytes except neutrophils (99). The agent is thought to inhibit migration of T lymphocytes and other peripheral blood mononuclear cells (PBMCs) across vascular endothelial cells and could thus interfere with lymphocyte-mediated immune surveillance within the central nervous system (CNS). Natalizumab has also been shown to reduce numbers of B lymphocytes in CSF, which could affect antibody-mediated control of JCPyV replication within brain parenchyma but could conceivably also reduce numbers of JCPyV-infected B cells entering CSF and brain. Other agents associated with PML have included rituximab (100), alemtuzumab, brentixumab vedotin, mycophenolate mofetil, and, more recently, dimethyl fumarate (Table 3) (101, 96). The effects of each of these agents on JCPyV persistence and reactivation have not been defined. Serum and JCPyV antibody levels in patients treated with natalizumab may have value in predicting patients who will develop PML (102), as may CSF anti-JCPyV antibody index (103, 104).

Association of BKPyV with Renal and Other Urological Diseases

BKPyV replication with high-level viruria and viremia is more prevalent in children or adults undergoing kidney or hematopoietic stem cell transplantation and may progress to tubulointerstitial nephropathy (105, 106, 107, 108), and/or hemorrhagic cystitis (7, 109, 110, 111, 112, 113). BKPyV-associated nephropathy affects 1% to 15% of kidney transplant recipients. Although the intensity of immunosuppression unquestionably plays a role in development of nephropathy, it is clearly not sufficient since BKPyV-associated nephropathy is rare in nonkidney solid organ transplantation receiving similar or even higher immunosuppression. Other risk factors include HLA-mismatches, older age and male sex of the recipient, transplantation of a graft from a seropositive donor with high antibody titers into a recipient with low or no antibody titers, cumulative steroid exposure, and tacrolimus-mycophenolate versus cyclosporine or mTOR inhibitor combinations. In rare cases, BKPyV nephropathy may be associated with congenital immunodeficiency (114, 115) or with HIV infection (116). BKPyV-associated hemorrhagic cystitis affects 5% to 20% of human stem cell transplant (HSCT) patients (7, 110, 111, 113). Risk factors include unrelated donor, myeloablative conditioning with urotoxic drugs including cyclophosphamide and busulphan, total body irradiation, and graft versus host disease. At present, BKPyV-associated urothelial carcinoma is a rare condition in immunosuppressed transplant patients and has occasionally been linked to chromosomal integration of the viral genome (117, 118).

Merkel Cell Carcinoma

MCPyV, like all other human polyomaviruses, is a ubiquitous and normally benign agent, without associated symptomatic infection (119). However, the virus is associated with Merkel cell carcinoma (MCC), a highly aggressive skin neoplasm first discovered in 1980, with an incidence of 0.24 per 100,000 patient years (120). MCC is predominantly a condition of the elderly and is most common under conditions of immunosuppression (120). Merkel cell carcinoma represents the only human condition in which clonal integration of a polyomavirus has been definitively associated with human cancer (121). The disorder is infrequent, with roughly 1,500 cases being reported per year but has a median survival of 9 months (119).

Trichodysplasia Spinulosa

Trichodysplasiaspinulosa (TS) represents a rare, potentially disfiguring cutaneous condition caused by TSPyV infection (69). The majority of cases have been reported in immunosuppressed individuals undergoing organ transplantation; a minority of cases have involved patients undergoing treatment for chronic lymphocytic leukemia (69). TSPyV is detected more frequently on the skin of HIV-positive than HIV-negative males but, somewhat surprisingly, the actual disorder has not as yet been reported to be increased in patients with HIV infection (122).

PATHOGENESIS

Animal Models of Human Polyomavirus Infection

None of the human polyomaviruses appear capable of causing productive infection in nonhuman species. For this reason, much of the current knowledge about the biological behavior of polyomaviruses in their natural hosts comes from studies of nonhuman agents. SV40 produces renal involvement in its simian host, as do JCPyV and BKPyV in humans. In immunocompromised animals, SV40 has been associated with fatal interstitial pneumonia, renal tubular necrosis, meningoencephalitis, and a PML-like illness (4, 123, 124). Under experimental conditions, mouse polyomavirus (MPyV) produces a protracted, widely disseminated infection followed by a latent renal infection that can be reactivated by pregnancy (4, 125).

The polyomavirus most extensively studied as a possible model for human infection is mouse pneumotropic polyomavirus (MPtV), formerly known as K virus. Infection is acquired by ingestion of virus, with initial replication of virus in intestinal endothelial cells (126, 83). In mice less than 6 days of age, MPtV produces an overwhelming infection of systemic and pulmonary vascular endothelial cells; death results from interstitial pneumonia (126). Older animals also develop widespread infection of vascular endothelial cells but survive acute infection. By 2 to 3 months following initial infection, viral nucleic acids, T-Ag, and V antigens can be detected in renal tubular epithelial cells, suggesting that viral persistence involves not actual latency but rather a chronic, low-level productive infection, similar to that now believed to occur in humans (127, 128) (Figure 6). Initial protection against lethal infection depends on a

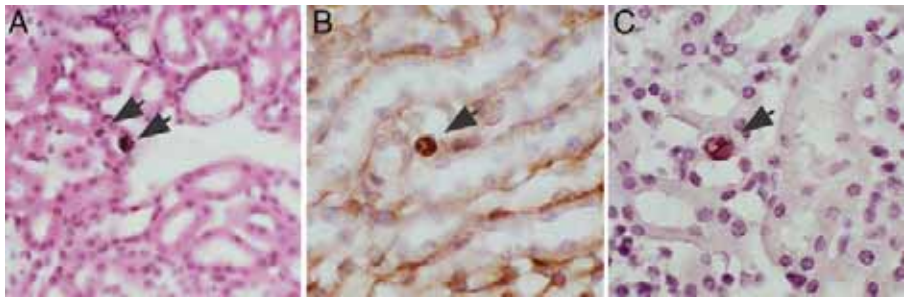

FIGURE 6 Polyomavirus persistence as a chronic productive infection, as shown in a kidney of a nonimmunosuppressed mouse 6 months after inoculation with murine pneumotropic (K) virus. Arrows indicate the presence of viral nucleic acids (A), T-Ag (B), and VP1 antigen (C) indicative of viral replication in renal tubular epithelial cells.

prompt antibody response. Cell-mediated immunity appears to be required for containment of persistent infection, and failure of T-lymphocyte function allows progression of infection by cell-to-cell spread (128).

With the exception of PML in simian AIDS, PML has not been observed in animals, nor have most attempts to produce PML in genetically modified animals been successful (129). Recently, CNS infection, with demyelination, has been produced following JCPyV inoculation into the brains of human glial chimeric *Rag2–/– Mbp*$^{shi/shi}$ mice (130). Although this model reproduces several features of PML, it differs from the disease seen in humans in that the model involves productive infection of astrocytes, an infrequent finding in human PML; also, oligodendrocyte death in this model involves apoptosis rather than the productive, lytic infection seen in humans. Attempts to reproduce human JCPyV infection in nonhuman species, using either JCPyV T-Ag or purified virus have resulted in dysmyelination in JCPyV T-Ag transgenic mice or in induction of a variety of tumor types in a number of species. However, none of these efforts has produced lesions resembling human PML (129).

Viral Replication and Persistence

Although none of the human polyomaviruses has been associated with symptomatic acute infection, primary infection by JCPyV or BKPyV is thought to result in viremia and dissemination of infection to organs distant from the site of initial exposure in particular the renourinarytract (131). TSPyV and MCPyV genomic sequences have been detected in urine, kidneys, lung, and blood, suggesting that these agents are also capable of causing disseminated infection (89, 132). Both JCPyV and BKPyV have been shown to persist in renal tubular epithelial cells (11). In addition, however, JCPyV DNA sequences have also been identified in multiple other organs from individuals without PML (133–136). Both archetypal and variant forms of JCPyV DNA have been detected in the brains of up to 33% of individuals without HIV infection or PML (137–139). These findings suggest that JCPyV may persist in the brain and that PML might be a consequence of reactivated infection within the CNS. It is not known whether persistence of these agents represents truly latent infection as opposed to productive infection at very low levels, controlled by host immune response.

Following initial detection of JCPyV DNA by *in situ* hybridization in B lymphocytes within the spleen and bone marrow of an AIDS patient with PML (140), a number of studies using PCR methods have detected JCPyV in PBMCs or other leukocytes (141, 142), suggesting that infection of circulating blood leukocytes could provide a mechanism for viral entry into the brain. Detection of JCPyV DNA sequences has been reported in PBMCs from 28.9% of 157 HIV-positive patients and from 16.5% of HIV-negative patients (143). In contrast, JCPyV DNA sequences both for the noncoding region of the genome and for VP1 were detected in only 0.9% of blood donors (144). In another study, DNA encoding JCPyV T-Ag was detected in blood from 31.8% of 60 AIDS patients but only in 2.3% of 88 immunologically normal blood donors (145). mRNA sequences encoding JCPyV VP1 were found in 38% of JCPyV DNA-positive urine samples from AIDS patients but in blood from only one individual (145). Other investigators have detected JCPyV DNA in T lymphocytes, monocytes, and polymorphonuclear leukocytes (146). In summary, these studies confirm that JCPyV may infect B lymphocytes and that the frequency of infection may be increased by immunosuppression. However, the studies also suggest that blood leukocyte infection by JCPyV may be latent and that other leukocyte populations may also support viral persistence, making it uncertain to what extent these cells are involved in dissemination of viral infection (139).

BKPyV is known to persist in kidneys. Infection of other organs and tissues is exceptional and essentially always associated with inherited immunodeficiency, immunosuppression for transplantation, or HIV-AIDS. In these settings, BKPyV has been detected in lungs, eyes, and brain (147–150).

MCPyV DNAs are readily recovered from normal skin, as are TSPyV, HPyV6, HPyV7, and HPyV9 (89), and studies employing next-generation sequencing may identify a wide diversity of polyomavirus species (151). MCPyV DNA has also been amplified from mucosal surfaces, and MCPyV DNA recovery has also been reported from internal organs, buffy coat preparations, and peripheral blood monocytes (132, 152). TSPyV is readily amplified from plucked eyebrows but has also been detected in nasopharynx, urine, and stool (69).

Reactivation of Infection

Earlier studies of persistent infection by JCPyV or BKPyV strongly associated reactivation or urinary excretion of JCPyV or BKPyV with immunosuppression. In these studies, serologic evidence for reactivation of BKPyV was detected in 22% to 44% of individuals undergoing renal transplantation or chemotherapy for malignant disease (65). Further, longitudinal studies of HIV-infected patients documented urinary excretion of BKPyV in 37% of patients and JCPyV in 22% (153). Urinary excretion of either virus was more likely in patients with low CD4 counts or low β_2-microglobulin

levels and did not correlate with serologic markers for either virus. Excretion of JCPyV did not appear to increase the likelihood of developing PML (153).

More recent studies employing PCR methods demonstrate that asymptomatic urinary excretion of JCPyV and BKPyV is common in healthy individuals and is unaccompanied by a rise in antibody titers. Urinary excretion of JCPyV DNA has been shown to occur in up to 62% of normal individuals (154, 63). In most studies, excretion of BKPyV has been less common, occurring in roughly 5% of patients (154, 63). However, one study has identified urinary excretion of BKPyV in 55% of premenopausal women, without detectable variation in viral excretion throughout the menstrual cycle (155), and another study detected urinary excretion of BKPyV DNA in 24% of children under 9 years of age, possibly reflecting excretion during primary infection (156). BKPyV DNA excretion fell to under 20% until age 30 and then gradually increased, reaching 44% by age 80 to 89 (156). Urinary excretion of JCPyV DNA was less than 10% in children 0 to 9 years of age and then steadily increased thereafter, reaching 75% by age 80 to 89 (156). Other studies have confirmed high rates of JCPyV DNA tend to be continuous, whereas excretion of BKPyV DNA appears to be more sporadic (154). These data plus older studies would suggest that urinary excretion of JCPyV or BKPyV, lasting days to weeks, is a recurrent event in many immunologically normal individuals, but that excretion of actual infectious virus is increased under conditions of immunosuppression. JCPyV and BKPyV DNAs recovered from nonimmunocompromised patients have been almost invariably archetypal (63), whereas JCPyV genotypes recovered from immunocompromised patients may consist of both archetypal forms and rearranged variants (11).

Reactivation of BKPyV or JCPyV infection, with urinary excretion of virus, usually as the archetype, is a common event during pregnancy in most women (157, 158). Rise in antibody titers indicative of reactivated JCPyV or BKPyV infection occurs in approximately one-third of pregnant women, most frequently at the end of the second trimester and irrespective of initially high antibody titers (157). Urinary excretion of BKPyV DNA may also be increased in pregnant women compared to healthy controls, with most individuals excreting the archetype (158, 159). Some, but not all, studies have detected increased excretion of the JCPyV archetype. Although reactivation of most of the newer polyomaviruses during pregnancy has not been studied, limited data suggest that urinary excretion of WUPyV, KIPyV, or HPyV9 is not increased during pregnancy (158).

Whether reactivation of human polyomavirus infection during pregnancy might result in infection of the fetus is debated. Although one group has reported detection of JCPyV IgM and BKPyV IgM in newborn infants (160), this has not been a consistent observation (161), and attempts to detect polyomavirus nucleic acid sequences in aborted fetuses or placentas have been inconsistent (162, 163, 164).

Immune Responses to Polyomavirus Infection

Polyomavirus infections elicit both antibody- and T-cell-mediated immune responses (11, 165, 166). The respective roles of the B- and T-cell responses in control of primary infection are not well understood. Impairment of the T-cell-mediated immune response results in increased urinary excretion of JCPyV or BKPyV despite continued high levels of circulating antibody, similar to the effect of immunosuppression on persistent MPtV infection in mice (167). Impaired T-cell function is almost invariably present in individuals succumbing to PML or BKPyV-associated nephropathy (168–174), and are presumed to play a role in the development of Merkel cell carcinoma, or *trichodysplasia spinulosa* (11, 89).

Pathogenesis of PML

Pathology

PML may involve cerebrum, cerebellum, or brainstem; the spinal cord is only rarely involved (4, 175, 176). Cerebral cortex and deep gray matter appear normal, but areas of retraction within subcortical or deep white matter indicate myelin loss. Histopathological examination of PML lesions demonstrates loss of oligodendrocytes in demyelinated areas, and remaining oligodendrocytes may have enlarged nuclei or contain actual intranuclear inclusions (4, 175). Astrocytes in and around PML lesions frequently develop hyperchromatic or multiple nuclei and mitotic figures (Figure 7) (4, 175). JCPyV nucleic acids and early and late viral proteins are present within nuclei of infected oligodendrocytes (4) (Figures 7 and 8). Atypical astrocytes in PML contain viral nucleic acids, but only occasionally express early or late viral proteins (Figures 7 and 8) (4, 177, 178). Electron microscopic examination of PML brains demonstrate crystalline arrays of viral particles within infected oligodendrocytes. Small numbers of viral particles can be found in occasional morphologically normal astrocytes but not within atypical astrocytes (4). Myelin breakdown and lipid-laden macrophages become evident as the disease progresses. In non-AIDS PML, extensive inflammation is unusual, although small numbers of lymphocytes may be seen around vessels and in demyelinated areas. Demyelination in AIDS-PML is often more extensive than in non-AIDS cases (179), and brains may contain areas of actual necrosis (180, 179). Lymphocytic perivascular infiltrates are more evident (180, 179), and may at times be accompanied by parenchymal infiltrates, which may include macrophages and multinucleated giant cells (179). Although atypical astrocytes have been considered a pathological hallmark for PML, these cells are often infrequent or absent in AIDS-PML brains and may also be rare or absent in non-AIDS cases. Lesions in PML accompanied by immune reconstitution inflammatory syndrome (PML) may lead to actual cavitation and show

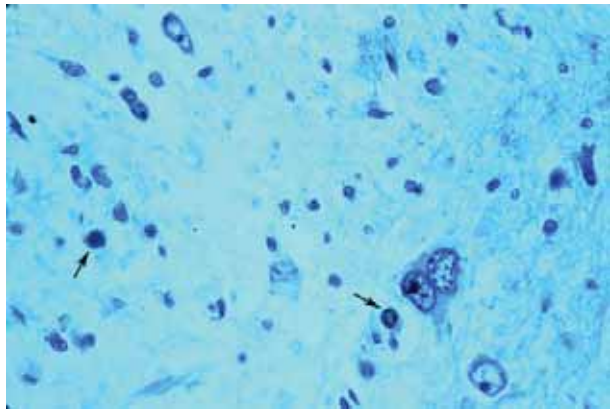

FIGURE 7 Section from edge of a PML lesion, stained for polyomavirus common structural antigen, and labeled using immunoperoxidase techniques. There is extensive loss of myelin. Oligodendrocytes have enlarged nuclei that exhibit intense immunostaining indicating productive infection (arrows). Giant astrocytes within the lesion remain unlabeled. Magnification, ×216.

FIGURE 8 Section of PML-affected brain probed for JCPyV nucleic acids using *in situ* hybridization methods. Large numbers of exposed emulsion grains, indicative of specific hybridization, overlie nuclei of infected oligodendrocytes. Smaller numbers of exposed emulsion grains overlie atypical astrocytes, again indicating presence of viral nucleic acids. Magnification, ×400.

extreme lymphocytic infiltration consisting of CD8+ and/or CD4+ lymphocytes and macrophages (181). Cases of PML in remission, an event that was essentially unknown before the late 1990s, may show persistence of viral antigen in areas of prior involvement (182). Although JCPyV infection of brain has been tightly associated with infection of oligodendrocytes and astrocytes, the virus can also produce productive infection of cerebellar granule cells or cortical neurons (183, 184).

Immune Responses

PML almost always occurs in the setting of immunosuppression, involving both T-cell-mediated and, to a lesser extent, antibody-mediated immune responses (4, 139). Abnormalities of T-cell response are essentially universal in patients with PML. Patients with non-AIDS PML have demonstrated a general impairment of T-cell-mediated immunity, with selective impairment of JCPyV T-cell responses (4, 139). The presence of JCPyV-specific cytotoxic CD8(+) T cells has been associated with better prognosis in PML, including those patients who develop immune reconstitution inflammatory syndrome (185, 186). In one series, 9 of the 10 patients whose PML entered remission following cART had positive CDA T cell responses, and restoration of JCPyV-specific CD4 T-cell responses was associated with clearance of JCPyV DNA from the CSF (187). In another study comparing prospectively identified PML survivors with nonsurvivors and CD4 matched controls for either group in the setting of HIV-AIDS, survival was linked to higher antibody titers and JCPyV-specific T-cell responses (174). This and other studies suggest that individual gaps in JCPyV-specific humoral and cellular immune response might explain why some patients succumb to PML, whereas others do not despite similar overall immunologic risk factors (174, 188).

Although patients with both AIDS-PML and non-AIDS PML may mount both systemic and intrathecal antibody responses to JCPyV, this response may not prevent the onset of disease. A rise in anti-JCPyV antibody response may occur before the onset of PML (189, 102, 174), and an intrathecal antibody response occurs in up to 76% of patients with PML (190). On the other hand, a rise in antibody titers may also accompany recovery, and a rise in titers of antibody to JCPyV has been observed during immune reconstitution in patients receiving cART (191, 174, 104).

Despite the fact that large numbers of individuals are persistently infected with JCPyV, PML is an unusual event, even in AIDS or during immunosuppressive treatment. This discrepancy suggests that compromised immune status alone does not account for the development of disease. The detection of JCPyV DNA in brains of immunologically normal patients suggests that PML might arise from reactivation of infection latent in the CNS (137, 138, 139, 136). It is possible that individual gaps in the JCPyV-specific immune response account for progression to PML and closing these gaps may be critical for disease prevention or survival outcome (174). Alternatively the frequent detection of JCPyV DNA in PBMCs from AIDS or other immunocompromised patients, with the caveats discussed above, could also provide a mechanism for viral entry into the brain.

Virological Features

An important question concerning the pathogenesis of PML has to do with the nature of the infectious virus. Archetypal JCPyV is at best rarely associated with PML, and most PML isolates have had NCRR duplications, deletions, or rearrangements. Although these genomic changes have been shown to alter the behavior of JCPyV in cell culture, their role in causing PML has not been determined (192, 139). JCPyV tropism for cerebellar granule cells, however, appears to correlate with a unique deletion in the region of the VP1 gene corresponding to the C terminus (193), suggesting that the deletions or rearrangements seen in the genomes of PML-associated JCPyV genotypes may have pathogenic importance.

In AIDS, pathological studies have documented a close association between AIDS PML lesions and HIV-infected T-cell/macrophage infiltrates, and large amounts of HIV antigens have been identified in some AIDS PML lesions (180). A direct facilitating role for HIV Tat or other proteins has been suggested by pathological and virological studies implicating *Tar*-sequences in the JCPyV archetype NCCR, which may no longer be necessary following NCCR rearrangements (194, 195, 192). Although PML has been reported in patients with a number of congenital immunodeficiency syndromes, no clear genetic association leading to PML *per se* has been reported (196). The association of PML with newer monoclonal immunomodulatory and other immunosuppressive agents has been discussed above.

Pathogenesis of BKPyV-Associated Urinary Tract Disease

Pathology

The finding associated with BKPyV excretion (and occasionally JCPyV excretion) under conditions of immunosuppression is the excretion of decoy cells (Figure 9), and of Haufen bodies, which represent clusters of cells expressing LTag (197). Decoy cell inclusions typically contain BKPyV virions, although cells infected by JCPyV may contain similar inclusions. In cases of ureteral stenosis, BKPyV-infected cells may be numerous enough to cause luminal narrowing or actual occlusion (198).

The characteristic pathological changes in the kidneys of individuals developing BKPyV-associated nephropathy are thought to progress through three stages, beginning with viral cytopathic effects (Stage A), cytopathic-inflammatory changes (Stage B), and tubular atrophy with interstitial

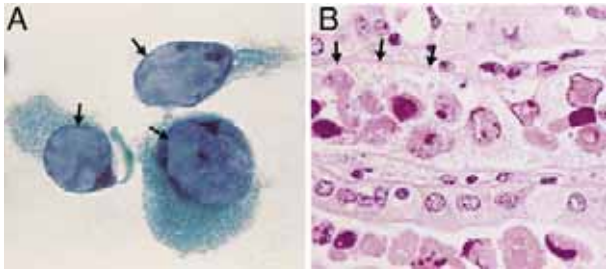

FIGURE 9 Urine and allograft-biopsy specimens from a patient with BKPyV-associated nephropathy. (A) Urine cytology showing "decoy cells" with their characteristic ground-glass, intranuclear viral inclusion bodies (arrows) (Papanicolaou stain, ×400). (B) Allograft-biopsy showing renal tubules with epithelial cells containing viral inclusions, nuclear enlargement, and detachment of infected cells from the tubular basement membrane, leading to its denudation (arrows) (hematoxylin and eosin, ×160). Adapted from reference 107 with the permission of the publisher.)

fibrosis (Stage C) (Figures 9 and 10) (198). In the few biopsied cases of hemorrhagic cystitis, BKPyV replication has been demonstrated by LTag staining together with inflammatory cell infiltrates. Clinical studies suggest the role of urotoxic drugs administered as part of the myeloablative conditioning as contributing factors to the urothelial damage (113).

Hemorrhagic cystitis may occur together with urethritis with or without ureteral obstruction, or less frequently, with interstitial nephritis (110, 113). Although most patients with hemorrhagic cystitis after allogeneic HSCT are seropositive, children with low or negative serostatus have been shown to develop hemorrhagic cystitis implicating nosocomial or even primary infection (112).

Immune Responses

The conditions that predispose to BKPyV nephropathy following renal transplantation are complex and involve not only host and viral factors but also presence of BKPyV in the donor kidney, HLA-mismatches, and types of treatment used post transplantation (199–201). Recent clinical and virological studies suggest that immunosuppressive drugs may have different direct effects on BKPyV replication leading to higher numbers of viremic patients (202, 203). JCPyV-associated nephropathy differs in that JCPyV viremia is usually low or undetectable, despite high urine viral loads and the presence of "decoy cells" (199).

Virological Features

The major known site of BKPyV persistence is the renal tubular epithelium. However, latent infection and low-level replication of BKPyV cannot be reliably distinguished, and it is not known to what extent the virus exists in a truly latent state or is maintained as productive infection at low levels involving cell-to-cell spread. Clinical disease can be caused by BKPyV replication in the tubular epithelial cells of the

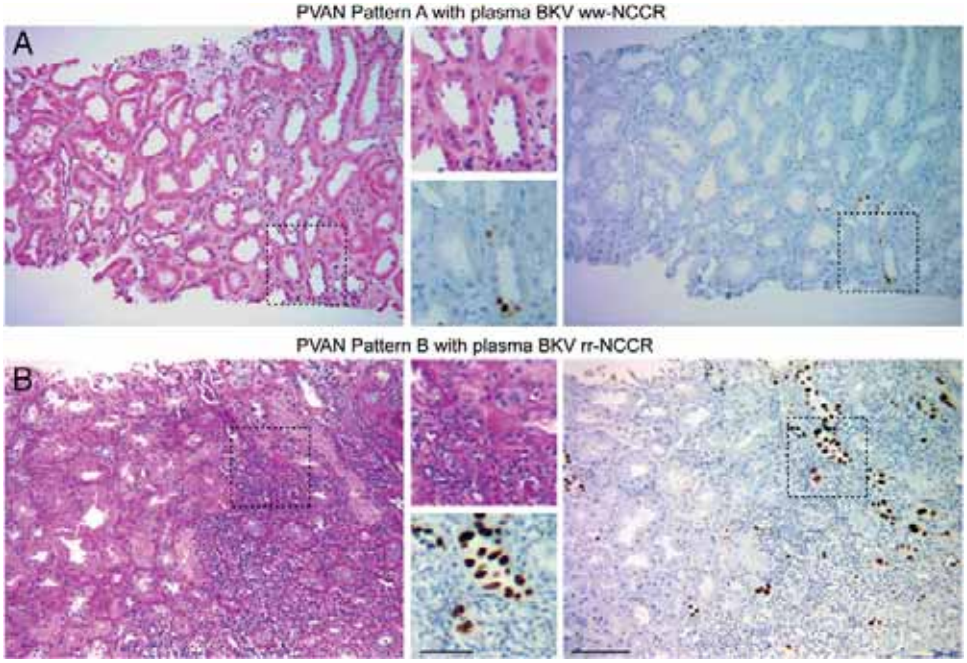

FIGURE 10 Histological patterns of polyomavirus-associated nephropathy (PVAN) in kidney transplant biopsies. (A) BKPyV nephropathy pattern A consisting of focal viral cytopathic changes with little inflammation or tubular atrophy of a patient with a BKPyV archetype NCCR in plasma. Hematoxylin/eosin stain of a representative histological field and immunohistochemistry of LTag expression using the cross-reacting monoclonal anti-SV40 T-antigen Ab-2 and peroxidase-conjugated anti-mouse IgG. Enlargement of the indicated area is shown. (B) BKPyV nephropathy pattern A consisting of extensive viral cytopathic changes and inflammatory infiltrates of patient carrying BKPyV rearranged (rr-)NCCR variant in plasma. Hematoxylin/eosin stain of a representative histological field and immunohistochemistry of LTag expression using the cross-reacting monoclonal anti-SV40 T-antigen Ab-2 and peroxidase conjugated anti-mouse. Bars: (A and B) 200 μm; (A and B, insets) 100 μm. Reprinted from Gosert R, Rinaldo CH, Funk GA, Egli A, Ramos E, Drachenberg CB Hirsch HH. 2008. Polyomavirus BK with rearranged noncoding control region emerge in vivo in renal transplant patients and increase viral replication and cytopathology. *J Exp Med* **205(4):**841–852 with the permission of the author and publisher.

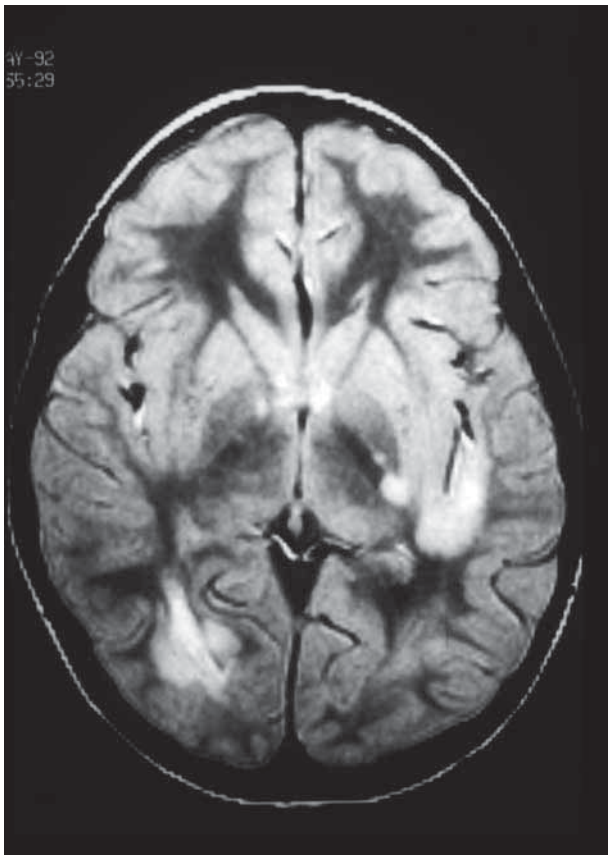

FIGURE 11 Fluid-attenuated inversion recovery (FLAIR) weighted magnetic resonance image of a patient developing PML in the setting of AIDS. Multifocal areas of demyelination are seen as areas of increased signal, appearing white against the darker background.

kidneys, the urothelial lining of the ureters, and the bladder. In BKPyV-associated nephropathy, however, the entire viral replication cycle has been documented, with initial detection of LTag in renal tubular epithelial cells followed by the expression of late VP1 and agnoprotein, consistent with cell-to-cell of infection within individual nephrons (172, 204). BKPyV replication in urothelial cells may play a role for disease progression via retrograde spread within nephrons in addition to hematogenous seeding through viremia (205, 198, 201, 206).

Pathogenesis of Merkel Cell Carcinoma and Trichodystrophy Spinulosa

MCPyV DNA has been detected in approximately 80% of tumors and found to be clonally integrated in cellular DNA of both primary tumors and metastases (132). Although development of Merkel cell carcinoma occurs in patients with impaired immune response, the malignancy is rare, even among severely immunocompromised patients. Merkel cell carcinomas express LTag. Seroreactivity to MCPyV LTag, as opposed to antibodies to MCPyV V1 antigen, is rarely seen in normal individuals but is seen at high titer in patients with Merkel cell carcinoma. MCPyV genomes isolated from MCC tissues harbor point and other mutations that result in expression of prematurely terminated LT (LTtrunc) lacking C-terminus sequences that are growth-inhibitory. It is thus of note that MPyV intact LTag and sTag sequences transform cells *in vitro* far less readily than do LTtrunc and sTag, which is consistent with observations that Merkel cell carcinomas express high levels of both LTtrunc and sTag (132). The interplay between impaired host antibody-mediated and T-cell-mediated immune responses, alterations in viral genomic function, and induction of neoplasia remains poorly understood (132).

As with MCC, trichodystrophy spinulosa is uncommon, associated predominantly with immunosuppression for organ transplantation or in patients with acute or chronic lymphocytic leukemias. At present the molecular and host events leading to the onset of trichodystrophy spinulosa are not understood. In contrast to the nonproductive MCPyV infection detected in Merkel cell carcinomas, the TSPyV infection leading to trichodystrophy spinulosa is productive, with expression of late viral proteins and production of actual virions (69).

Carcinogenesis

Murine polyomavirus was discovered through its ability to produce tumors following inoculation into its natural host, and SV40, BKPyV, and JCPyV had been shown before 1980 to cause central nervous system tumors in experimental animals (4, 207). For this reason there has been long-standing interest in whether or not polyomaviruses might also cause tumors in humans. To date, however, no human polyomavirus other than MCPyV has been definitively linked to a human tumor. Tumor production by any of these viruses in animals usually requires conditions such as the use of large quantities of virus, inoculation into species other than its natural host, and inoculation under conditions of compromised immune status or into sites such as the CNS, which are relatively sequestered from host immune response. In the case of JCPyV, the most suggestive evidence that the virus might cause tumors in humans lies in 3 reported cases characterized by multifocal gliomas abutting PML lesions (208–210). However, none of these cases has been studied using modern molecular techniques. An important area of ongoing research has been the possible causative role of BKPyV in bladder or other urinary tract carcinomas in solid organ transplant (SOT) patients, a group of patients at high risk for productive BKPyV infection (118). In 20 patients reported to date, clonal expression of polyomavirus large T-antigen or BKPyV DNA, but not cytopathic effects indicating viral replication, has been detected in these tumors and their metastases (118). Similar clonal expression of large T-antigen has also been detected in tumors arising in native and transplant kidneys and ureters (118). Chromosomal integration of BKPyV has been demonstrated in one case (117).

JCPyV, BKPyV, and, in particular SV40 antigens or DNA have been detected by immunohistological or PCR methods in a number of human tumors; including choroid plexus papillomas and other brain tumors, mesotheliomas, colonic and esophageal tumors, non-Hodgkin's lymphomas, and prostate tumors. However, detection of viral DNA or antigens within a tumor does not necessarily indicate causation and could be explained by one of two other scenarios (207). First, the virus could be a "passenger," meaning that it finds favorable conditions for replication in an already transformed cell. Second, the virus could be present not within the tumor itself but in nonneoplastic adjacent cells or tissue compartments (207). In addition, absence of viral DNA from a tumor could also be the result of a "hit-and-run" process in which malignant transformation was initially

caused by the virus, but the virus is no longer detectable after transition of the cell to a fully malignant state (207). An additional complicating factor is that polyomaviruses are extremely hardy, and viral DNA can be assumed to be a potential contaminant in any laboratory in which the virus has been handled. In general, confirmation of polyomavirus association with individual tumors—other than MCPyV—has not been confirmed in independent laboratories; and studies employing improved PCR methods and more stringent controls have also failed to confirm most earlier reports identifying polyomavirus DNA in human tumors. The association of BKPyV and neoplasia is particularly perplexing in prostate neoplasms: studies employing high-throughput RNA sequence (RNA-Seq) methods have failed to detect BKPyV nucleic acids in a total of 213 prostate cancers or in 39 nonmalignant prostate samples (211–213). These observations could be consistent with a "hit-and-run" hypothesis of viral causation but could also indicate that BKPyV had no role in these neoplasms. A more recent study showed that higher antibody titers to the common N-terminus shared by LTag and sTag were associated with a better prognosis of prostate cancer and improved the predictive value of tumor stage, Gleason score, and surgical margin status (214).

SV40 has been postulated to cause human neoplasia since its discovery as a contaminant in batches of poliovirus and adenovirus vaccines, and detection of SV40 sequences in human tumors is still being reported and discussed (213, 215). Studies of army veterans exposed to SV40 in contaminated adenovirus or poliovirus vaccines have not shown an increased incidence of cancer over time (216). Some SV40 isolates have been found, upon careful examination, to contain plasmid DNA sequences indicating laboratory contamination (217). Other isolates have been shown to be 776-SV40, a widely used laboratory strain that has never been directly isolated from monkeys and that may also represent contamination rather than natural infection (14). Only one group of investigators has attempted to duplicate PCR amplification of polyomaviruses from the same tumor material in two geographically separated laboratories (218). Although JCPyV, BKPyV, or SV40 DNAs were detected in a small number of cases, neither laboratory could confirm the other laboratory's positive cases (218). At present, other than the possible association of BKPyV with renourinary tumors in solid organ transplant patients, there is insufficient evidence to associate JCPyV, BKPyV, or SV40 with human neoplasia.

CLINICAL MANIFESTATIONS

Acute JCPyV or BKPyV infections are usually asymptomatic. One 13-year-old, immunocompetent girl was reported to have undergone seroconversion to JCPyV in association with chronic meningoencephalitis (219), and JCPyV DNA has been detected in the CSF from an individual with meningitis in the setting of systemic lupus erythematosus (220). BKPyV has been isolated from the urine of pediatric patients with both hemorrhagic and nonhemorrhagic cystitis. JCPyV and BKPyV DNAs were detected by PCR in 3.8% and 1.5% of 181 CSF samples from individuals with meningitis or encephalitis but not in 20 CSF samples from control subjects (221). In none of these reports, however, was a causative role for either virus proven, and two of the positive samples (one positive for JCPyV and one for BKPyV) were also positive for *Mycobacterium tuberculosis*, suggesting a much more likely cause of the neurological illnesses (221).

Progressive Multifocal Leukoencephalopathy

PML usually begins insidiously, with initial symptoms and signs suggesting focal cerebral involvement: these may include alterations in personality, changes in intellect, focal weakness, difficulty with motor skills, or sensory loss (4, 175). Involvement of the dominant cerebral hemisphere may result in expressive or receptive dysphasia. Visual field abnormalities, including actual cortical blindness, occur in 50% of patients (4). Occasionally, PML begins with signs of brainstem or cerebellar involvement, with difficulty in phonation, swallowing, abnormalities of extraocular movements, or ataxia (4). Spinal cord involvement is rare and is virtually never symptomatic (176).

Without intervention, the course of PML is remorselessly progressive. Initial symptoms are followed by the appearance multifocal neurological signs, increasing dementia, and progression to a vegetative state. Most patients with non-AIDS PML die within one year, but death may occur within as little as 2 months, and cases have been reported with survivals of 8 to 10 years or longer (4). Survival in untreated AIDS patients with PML averages 4 months. cART has significantly altered the frequency and course of AIDS-PML, so that the 1-year and 3-year survival rates for AIDS-PML are 55% and 50%, respectively, and 79% at 1 year for patients with CD4+ count above 100 cells/mm^3 at diagnosis (95). The Swiss HIV Cohort Study of 186 cases of PML, 159 of whom were diagnosed before death, demonstrated a significant reduction in mortality in patients starting cART compared to mono- or dual drug therapies (94). Survival in AIDS-PML is highly dependent upon adherence to cART (222).

Patients developing PML while receiving immunomodulatory or immunosuppressive agents may also stabilize or improve after withdrawal of the therapeutic agent. In patients developing PML following treatment with natalizumab and treated with withdrawal of medication plus plasma exchange and/or immunoadsorption, mortality is now 23% (223), although survivors may be left with significant neurologic deficits (223). Development of IRIS is an almost universal concern in these patients (224); and onset of PML-IRIS *de novo* has been reported after discontinuation of natalizumab (225).

Clinical Diagnosis

PML should be considered in any immunocompromised patient who develops progressive neurological deficits involving multiple areas of brain (4). Patients with untreated HIV and patients receiving natalizumab should be considered at high risk. Hematological studies and blood chemistries are unhelpful in the diagnosis of PML. Cerebrospinal fluid is usually normal but may occasionally contain increased protein or, rarely, a lymphocytic pleocytosis (4).

The most useful screening study for PML is magnetic resonance imaging (MRI) (4), which will show altered signal in subcortical and deep white matter on T2 and FLAIR images (Figure 11). Some, but not all, PML lesions enhance with intravenous gadolinium. MRI should be followed by analysis of CSF for JCPyV DNA (226). A particular challenge in dealing with patients with multiple sclerosis is the differentiation of demyelination due to PML from that due to the underlying disease. MRI changes considered suggestive of PML include lesion size over 3 cm; subcortical localization; presence of lesions in the basal ganglia; hyperintensity on T2, FLAIR, and DWI images with hypointensity on T1 images; lesions that are sharp toward gray matter and ill-defined toward white matter, and a tendency

for lesions to increase in size and for new lesions to appear (227). Hyperintensity of lesions on T1-weighted images suggests PML-IRIS (227).

Several other infectious agents may mimic PML clinically in immunosuppressed patients. In patients with AIDS, these include central nervous system invasion by *Toxoplasma gondii*, *Cryptococcus neoformans*, or *Mycobacterium tuberculosis*. These infections may occur together with or in the absence of PML. Central nervous system lymphoma may also mimic PML, in particular in AIDS patients. Brain biopsy to rule out treatable conditions may thus be crucial in these patients where the diagnosis of PML is at all in question or where there is concern about other, coexisting conditions.

Other CNS Infections Associated with JCPyV

Although JCPyV has traditionally been thought to produce an infection limited to cells of glial origin—oligodendrocytes and astrocytes—it has also been associated with infection of cerebellar granule cell neurons and of pyramidal neurons, causing a cortical encephalitis. Granule cell neuronopathy has been described in AIDS patients and patients treated with monoclonal antibodies and may occur in the presence or absence of PML (228, 229, 183). In such cases, cerebellar atrophy and white matter changes may be detectable on MRI (229). Analysis of JCPyV DNA from the granule cells of one patient with coexisting PML revealed a unique deletion in the C terminus of the VP1 gene not found in JCPyV DNA present in the patient's white matter lesions, suggesting that the deletion, with its accompanying frame shift, may have enabled the virus to infect granule cell neurons (193). Unlike PML *per se*, granule cell neuronopathy may run a nonfatal course (183). Cortical encephalitis has been associated with a JCPyV genotype with a unique 143 bp deletion in the gene encoding the agno protein (230, 231).

BKPyV Renourinary Tract Infection

Urinary tract involvement by BKPyV may cause cystitis, ureteral obstruction, or nephropathy with stage B nephropathy previously being called interstitial nephritis. Nephropathy is most frequently seen following organ or stem cell transplantation and is seen in 4% to 8% of renal transplant patients (232, 65, 233). Rare cases of nephropathy have also occurred in children with severe immunodeficiency syndromes and in occasional patients with AIDS (234). In some cases, nephropathy may be accompanied by symptomatic cystitis or ureteral obstruction. More commonly, however, BKPyV-associated nephropathy is clinically silent and is manifested in 40% to 60% of cases by progressive renal failure (232, 65). In renal transplant patients, the condition may be confused with graft rejection (232, 65). The risk for BKPyV nephropathy following renal transplantation is known to be associated with a number of factors including high BKPyV antibody titers in the donor, low or absent BKPyV antibodies in the recipient, HLA-mismatches, and potent immunosuppressive therapy often involving tacrolimus-mycophenolate combinations (235, 236, 202). A minority of cases of posttransplantation nephropathy are associated with JCPyV (237–239). Ureteric stenosis due to BKPyV has become much less common today in kidney transplantation despite extensive urothelial replication, presumably due to improved surgical techniques and ureteric stent implantation.

Cystitis due to BKPyV may be hemorrhagic or nonhemorrhagic, occur during primary or reactivated infection, and be occult or symptomatic (240). In transplant patients, hemorrhagic cystitis associated with BKPyV usually occurs well after organ transplantation and should be differentiated from that seen soon after transplantation, which is more commonly due to cyclophosphamide or other immunosuppressive agents (65, 240). Hemorrhagic cystitis is, by definition, clinically symptomatic with urgency, dysuria, and macrohematuria (110, 113). The condition may occur together with urethritis with or without ureteral obstruction, or less frequently, with interstitial nephritis. Although most patients with hemorrhagic cystitis after allogeneic HSCT are seropositive, children with low or negative serostatus have been shown to develop hemorrhagic cystitis implicating nosocomial or even primary infection (112). The disease has to be differentiated from other etiologies including direct toxic effects, herpes simplex, cytomegalo-, and adenovirus infection (113).

Other Conditions Associated with BKPyV Infection

In a handful of cases, all of them in profoundly immunocompromised patients, BKPyV has been reported to cause interstitial pneumonia (241). In one renal transplant patient, BKPyV was associated with a widespread infection of vascular endothelial cells resulting in muscle weakness, anasarca, and myocardial infarction (242). BKPyV has been occasionally detected in the CSF of children with encephalitis (221) and was identified in CSF from one patient with frontal and temporal encephalomalacia in the setting of renal transplantation (243). BKPyV has also been associated with cases of retinitis and of meningitis or encephalitis but in most of these cases there has not been clear proof that BKPyV was the causative agent (241).

Merkel Cell Carcinoma and Trichodystrophy Spinulosa

Merkel cell carcinoma and TS are both conditions involving the skin, but with radically different clinical course, morbidity, and mortality. The tumor most commonly occurs on sun-exposed areas of skin as a small, cystic lesion that enlarges over weeks to months. MCC metastasizes to draining lymph nodes and also to brain, bone, and systemic organs. Mean survival in patients is under 6 months (120).

Trichodystrophy spinulosa is a rare skin condition, characterized by development of spiny follicular papules located predominantly on the face and, in a smaller number of cases, on extremities, trunk, or scalp. The disease has been observed under conditions of immunosuppression and occasionally in patients with chronic lymphocytic leukemia. The condition can be disfiguring but, in contrast to MCC, is not yet known to have appreciable mortality (69).

Manifestations of Other Polyomaviruses

Nine other human polyomaviruses have been identified (Table 1). Most if not all of these agents are widespread agents of human infection. To date, however, the majority of these agents have not been associated with clinical illness. HPyV6 and HPyV7 are shed from the skin of healthy subjects as well as patients with a variety of skin tumors. KI and WU viruses have been recovered from nasopharyngeal aspirates of children with respiratory tract infections and STLPyV has been recovered from tonsils of children with chronic tonsillitis. However, the actual role of these agents in disease causation has not been established. To date, of the newer polyomaviruses, the only one linked to clinical illness is NJPyV, which has been associated with microvasculitis of muscle and retina and with a necrotizing dermopathy in one pancreatic transplant patient (244). Pathological evaluation

of muscle in this patient revealed a vasculitis affecting small vessels. NJPyV DNA was recovered from both skin and muscle, and polyomavirus inclusions were found in vascular endothelial cells, similar to the vascular involvement seen with the mouse agent, MPtV (244, 127).

LABORATORY DIAGNOSIS

JCPyV Infection and PML

PML was initially a pathological diagnosis, with diagnostic accuracy enhanced by immunohistochemistry for JCPyV antigens or *in situ* DNA hybridization methods. At present, the diagnostic study of choice for PML is PCR analysis of CSF for JCPyV DNA (226). In earlier studies, use of PCR analysis of CSF enabled specific diagnosis in 80% to 90% of cases (4), and this level of sensitivity is still thought to apply in cases of non-AIDS PML, including cases associated with natalizumab and other agents affecting host immune response. In patients with AIDS-PML, however, cART has been shown to reduce viral load of JCPyV in CSF, so that diagnostic yield in patients treated with cART has been reduced to 57.5% (245). Cell culture isolation is extremely cumbersome and not used for diagnostic purposes.

BKPyV Infection

Renourinary infection by BKPyV may be suspected by the detection of decoy cells (urothelial cells containing nuclear inclusions) in urine (Figure 9). Urinary decoy cells, although highly specific, are seen in 40% to 60% of transplant patients but associated with nephropathy in only 20% of cases (232). Viremia with over 7,000 copies of BKPyV DNA per ml has a predictive value of approximately 60%, and urinary excretion of over 100 times the number of copies per ml in plasma has a predictive value of 40% (232). Although transplant biopsy remains the gold standard to diagnose BKPyV, quantitative analysis of urinary decoy cells and identification of urinary Haufen bodies provide good surrogate markers for surveillance (246, 247).

Serologic Assays for JCPyV and BKPyV

Antibodies to JCPyV and BKPyV can be measured by hemagglutination inhibition (248) or ELISA methods. Detection of antibodies to JCPyV or BKPyV is not itself diagnostic of disease, since most individuals have antibody. The presence of antibody does, however, indicate prior infection, and elevation of JCPyV antibody index in patients on natalizumab or other agents may suggest increased risk of PML. Severe immunosuppression may prevent a rise in antibody titer.

PREVENTION

Vaccines or other specific measures to prevent JCPyV or BKPyV infection have not been developed. In recent years, however, major attention has focused on the identification of individuals at increased risk of developing PML while under treatment with natalizumab or other immunomodulatory agents, or of developing BKPyV nephropathy and then adding risk stratification to alter their immunosuppressive regimens. The presence of antibodies to JCPyV has been intensively studied as a tool for risk stratification in individuals receiving natalizumab, based on findings that individuals who are seropositive for JCPyV appear to be at a higher risk for developing PML (102). Use of a commercially available JCPyV antibody index has demonstrated that natalizumab-treated patients developing PML maintain higher anti-JCPyV antibody index values over time than did patients not developing PML, suggesting that antibody index may provide a means of assessing PML risk in antibody-positive natalizumab-treated patients (102).

BKPyV-associated nephropathy is asymptomatic in almost all cases, and the disease is not recognized until renal function is significantly impaired. Therefore, current guidelines emphasize the role of screening for BKPyV in urine and blood (246, 247, 201). The paradigm of BKPyV reactivation after kidney transplantation proceeds from low-level viruria detected by quantitative PCR to high-level viruria with BKPyV loads above 7 $\log_{10}$ copies/ml and the appearance of "decoy cells" in 20% to 40% of patients (108, 249, 206). BKPyV-associated nephropathy is more likely if plasma viral loads are above 7,000 copies/ml (108, 198). Although transplant biopsy remains the gold standard for a proven disease, plasma viral loads above 10,000 copies/ml are widely accepted for the diagnosis of presumptive nephropathy and preemptive treatment, e.g., by reducing immunosuppression (201). Patients with high urine viral loads above 10 million copies/ml are considered to be at risk for viremia and nephropathy, but a preemptive treatment approach by immunosuppression may not be justified (249) as it unnecessarily increases the risk of precipitating rejection (250).

Treatment of PML

Attempts to treat PML with antiviral agents have employed cytosine arabinoside, camptothecin, topotecan, cidofovir, mefloquine, and serotonin receptor blockers such as mirtazapine, but no agent of proven value has been identified to date. Brincidovovir, a lipid conjugate of cidofovir (formerly called CMX001), has been found to inhibit JCPyV replication in human brain progenitor-derived astrocytes and human fetal brain SVG cultures but has not as yet been reported in clinical trial (251, 252). The use of cytosine arabinoside, cidofovir, camptothecin, and topotecan may be accompanied by significant toxicity. An uncontrolled study reported neurological stabilization or improvement in 7 of 19 non-AIDS PML treated with intravenous cytosine arabinoside at 2 mg/kg for 5 days (253), but controlled studies have shown no benefit in HIV-infected individuals (254). Clinical improvement has been described in individual AIDS and non-AIDS patients treated with cidofovir, including cases in which cidofovir was added to cART or other antiviral agents (255–258). However, efficacy has not been demonstrated in case series of AIDS-PML, and controlled trials have not been reported (259). Camptothecin and topotecan have been used in individual cases of PML, but therapeutic efficacy has not been confirmed (260). Mefloquine, which has been shown to have antiviral activity against JCPyV *in vitro*, has been used in individual patients, as has the serotonin reuptake inhibitor, mirtazapine, with or without concomitant treatment with cidofovir or mefloquine. Although individual case reports have suggested clinical improvement with these agents, mefloquine was not demonstrated to be effective in a controlled trial (261), and controlled studies of mirtazapine have not been reported. The use of monoclonal agents directed against JCPyV capsid proteins has been suggested but not yet used in clinical practice (262).

In contrast to the poor results seen with antiviral agents in treatment of PML, virological and clinical improvement have been definitively shown to occur in cases in which it is possible to restore host immunocompetence by treatment of AIDS patients with cART, removal of monoclonal agents,

such as natalizumab, by plasma exchange with or without immunoadsorption, or discontinuation of other immunosuppressive agents (95, 263). In such cases, restoration of immunocompetence may result in life-threatening IRIS, whose treatment may require corticosteroids or other measures (262); IRIS will occur in over 70% of cases of PML associated with natalizumab following discontinuation of medication (262).

Treatment of BKPyV Nephropathy

There is currently no specific antiviral therapy to treat BKPyV nephropathy. A placebo-controlled prospective randomized trial of levofloxacin prophylaxis for the first 3 months after kidney transplantation did not demonstrate lower rates of BKPyV viruria or viremia, but an increased risk of bacterial resistance (264). Monitoring of viral load and preemptive withdrawal of immunosuppression were associated with resolution of BKPyV viremia and decreased rates of nephropathy (249, 171, 265, 250, 236, 266). Multiple case series have suggested therapeutic benefit from cidofovir, but controlled trials demonstrating efficacy have not been published. Commercially available immunoglobulin G, which contains neutralizing antibodies to all BKPyV genotypes, has shown potent antiviral activity against BKPyV *in vitro*. In one study of 30 patients with BKPyV nephropathy unresponsive to cidofovir and use of leflunomide, treatment with IgG intravenously at 1g/kg produced clearing of viremia in 90% of patients and 12-month graft survival rates of 96.7% (267). Brincidofovir has shown a good inhibitory effect of BKPyV replication in tubular epithelial and urothelial cells *in vitro*, and it is currently in clinical trials (268–270).

Treatment of MCC and TS

Treatment of MCC may involve surgery, radiation treatment, and chemotherapy. Antiviral treatment has as yet no place in treatment of the neoplasm. Improvement in TS has been reported with withdrawal of immunosuppressive agents, and individual cases have been reported to improve with topical cidofovir or oral valganciclovir (271).

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Papillomavirus

WILLIAM BONNEZ

29

Human papillomaviruses (HPV) are a large group of viruses (more than 200 types identified) that infect the epithelium of the skin and mucous membranes. The infections can be latent, subclinical, or clinically manifest, causing lesions that range from the benign (warts, papillomas, condylomas) to the malignant. Some of these viruses are responsible for cutaneous warts; a different group of HPVs (types 6 and 11) is responsible for most genital warts. The major importance of HPVs is because 20 types (especially HPV-16 and -18) are regarded as the necessary, if not sufficient, cause of cervical cancer as well as other cancers of the vagina, vulva, penis, anus, and oropharynx. Although most sexually active individuals get infected with genital HPVs, most of these infections are transient and innocuous. It is the persistence of the high-risk oncogenic HPVs that leads to malignant transformation. Cancer caused by these viruses is preceded by a precancerous stage whose detection is the basis of cytologic screening (Pap smear) for cervical cancer. Ten years ago HPV vaccines were introduced for the prevention of diseases associated with HPV-16 and -18 and for the prevention of anogenital warts caused by HPV-6 and -11. The introduction of HPV vaccination has been a major advance because it is the first time a vaccine has been able to directly prevent virally induced cancers. When used broadly before the onset of sexual activity, HPV vaccination has caused a dramatic reduction in the incidence of genital warts and HPV-related cervical lesions. This should be the harbinger of a decline in the rates of cervical and other cancers.

HISTORICAL BACKGROUND

Hand and plantar warts and external anogenital warts, also called condylomata acuminata, have been known since antiquity. However, it was in 1871 that Mackenzie recognized the existence of laryngeal papillomas and in 1922 that Lewandowsky and Lutz described epidermodysplasia verruciformis (1, 2). Variot in France was the first to demonstrate the transmissibility of warts in 1891, while Ciuffo in Italy established their viral nature in 1907 (3, 4). Human papillomaviruses (HPVs), first identified by electron microscopy in 1949 by Strauss et al. (5), primarily infect stratified squamous epithelia of humans. The Crawfords showed that the virus had a double-stranded DNA genome (6). HPV was originally thought to cause only warts, but during the past 40 years the great biological importance of these ubiquitous viruses has become recognized. HPVs are, in fact, responsible for a wide array of diseases, both benign and malignant. Rarer diseases such as recurrent respiratory papillomatosis (laryngeal warts) and epidermodysplasia verruciformis were found in the late 1960s and early 1970s to be related to HPV infection. In 1974 the role of HPV in cancer of the uterine cervix was first suspected. This oncogenic potential had already been established for the cottontail rabbit (Shope) papillomavirus in 1932 and for bovine papillomaviruses (BPV) in 1951. In the 1970s, analysis of HPV isolates led to the recognition of multiple HPV genotypes by the groups of Gérad Orth at the Pasteur Institute in Paris and Harald zur Hausen in Freibourg and then Heidelberg (7–12). zur Hausen and his colleagues established an association between HPVs and squamous benign and preinvasive malignant lesions of the uterine cervix. These preinvasive malignant lesions, also called dysplasias, intraepithelial neoplasias, or squamous intraepithelial neoplasias (SIL), are the precursors to invasive cervical carcinoma. As studies on the biology of HPVs progressed, it became clear that some of these viruses possessed genes capable of transforming and immortalizing cells *in vitro*. Subsequent epidemiological observations showed that some genital HPV types, the "low-risk" HPVs (e.g., types 6 and 11), were never or rarely associated with malignancies, whereas others, the "high-risk" HPVs (e.g., types 16 and 18), were linked to cancers. Harald zur Hausen was awarded the Nobel Prize in Medicine or Physiology in 2008 for his original contributions to the association between HPV and cervical cancer. The use of polymerase chain reaction (PCR) assays to diagnose HPV infection in prospective epidemiological studies demonstrated that HPVs play a central role in the vast majority of cervical cancers. HPVs are also associated with many other cancers, with a causal link becoming increasingly established for squamous cell carcinomas (SCC) of the anus, vulva, vagina, penis, and oropharynx. In 2006 the first highly effective HPV vaccine directed at HPV type 6 (HPV-6), -11, -16, and -18 became available, followed in 2009 by a second, bivalent (HPV-16 and -18) vaccine. Already, substantial reductions in the burden of anogenital HPV infections and associated diseases have been observed in many countries. Anogenital warts are almost eradicated wherever the quadrivalent vaccine is being used. In 2015, a third, nonavalent vaccine (HPV-6, -11, -16,

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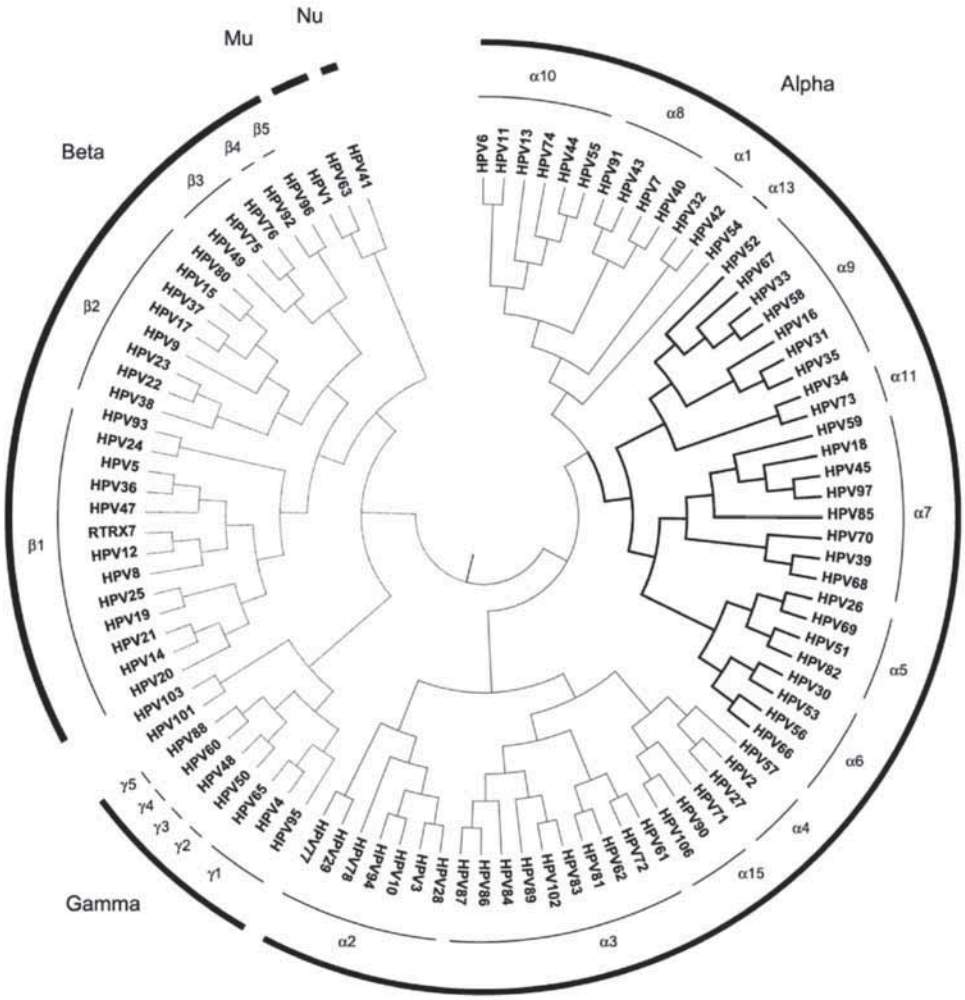

FIGURE 1 Phylogenetic tree of 99 human papillomaviruses, their genuses and species. The tree was established on the concatenated amino acids and nucleotide sequences of six open reading frames (E6, E7, E1, E2, L2, and L1) (reprinted from reference 2 with permission).

-18, -31, -33, -45, -52, and -58) was introduced to the market, which promises greater vaccination rate and an accelerated disappearance of the Papanicolaou (Pap) smear for the primary screening of cervical cancer.

VIROLOGY

Classification

All the members of the *Papillomaviridae* family, including HPVs, have a supercoiled, single double-stranded circular DNA genome enclosed in an unenveloped, icosahedral capsid (http://www.hpvcenter.se/html/refclones.html, http://pave.niaid.nih.gov/). Papillomaviruses are widespread among higher vertebrates but are species specific (13). Because HPVs could not be grown for many years, and because sufficient quantities of most of them cannot be purified for direct biochemical and antigenic characterization, HPVs are classified by genotypes and not serotypes.

Genotypes

Papillomaviruses are classified as distinct genotypes if they have less than 90% DNA sequence homology of the L1 open reading frame (ORF), which encodes the major capsid protein. Subtypes have between 90% and 95% DNA homology, and variants have between 95% and 98% DNA homology. The *Papillomaviridae* family has 49 genera at present (http://www.ictvonline.org). HPVs belong to the Alpha-, Beta-, Gamma-, Mu-, and Nupapillomavirus genera. Although papillomaviruses share the same general genomic organization, some particularities characterize each genus. For example, the Beta- and Gammapapillomaviruses lack an E5 ORF. A genus may be further divided into species defined by a particular representative. For example, HPV-16, an alphapapillomavirus, is the representative type of species 9, which also includes types 31, 33, 35, 52, 58, and 67 (Fig. 1) (14–17). At the end of 2015, 205 HPV genotypes had been formally identified, a number that is continuously expanding. Most recent types do not appear to be associated with any disease (Table 1). There is an imperfect concordance between the phylogeny of the HPV types and their biology. All the Betapapillomaviruses are associated with cutaneous lesions, with species 1 and 2 encompassing the epidermodysplasia verruciformis HPV types. The Gamma-, Mu-, and Nupapillomaviruses are all associated with cutaneous lesions. Among the Alphapapillomaviruses the situ-

TABLE 1 HPV types and their disease associations[a]

Disease	HPV type(s)	
	Frequent association[b]	Less frequent association
Cutaneous		
Deep plantar warts	1, 2, 27	4, 41, 57, 63
Common warts	2, 1, 4, 27	3, 7, 26,[c] 28, 29, 41,[d] 57, 65, 77,[d] 117,[c] 125, 128, 129 130, 131, 132, 133, 148, 149, 179, 184
Common warts of meat, poultry, and fish handlers	7, 2	1, 3, 4, 10, 28
Flat warts	3, 10	27,[c] 38, 41,[d] 49,[c] 75, 76
Epidermodysplasia verruciformis	5,[d] 8,[d] 9, 12, 14,[d] 15, 17[d]	19, 20[d,e] 21, 22, 23, 24, 25, 36, 37, 38, 47,[d] 49, 50, 75, 93
Anogenital or mucosal		
Condylomata acuminata	6, 11	42, 43, 44,[e] 45,[d] 51,[d] 54, 70, 153, 175, 178, 180
Intraepithelial neoplasia		
Unspecified		26,[d] 30,[d] 39,[d] 40, 53,[d] 57, 59,[d] 61, 62, 67,[d] 68,[d] 69, 71, 81, 83
Low grade	6, 11	16,[d] 18,[d] 31,[d] 33,[d] 35,[d] 42, 43, 44,[e] 45,[d] 51,[d] 52,[d] 54, 61, 70, 72, 74[c]
High grade	16,[d] 18[d]	6, 11, 31,[d] 33,[d] 34,[c,e] 35,[d] 39,[d] 42, 44,[e] 45,[d] 51,[d] 52,[d] 56,[d] 58,[d] 66,[d] 67[d]
Cervical carcinoma	16,[d] 18[d]	26, [d] 31,[d] 33,[d] 35,[d] 39,[d] 45,[d] 51,[d] 52,[d] 53,[d] 56,[d] 58,[d] 59,[d] 66,[d] 67,[d] 68,[d] 70,[d] 73,[c,d] 82[d]
Recurrent respiratory papillomatosis	6, 11	16,[d] 18,[d] 31,[d] 33,[d] 35,[d] 39[d]
Miscellaneous		
Focal epithelial hyperplasia	13, 32	18,[d] 33,[d] 45[d]
Conjunctival papillomas and carcinomas	6, 11, 16[d]	18,[d] 33,[d] 45[d]
Others, cutaneous[f]		36, 37, 38,[d] 41,[d] 48,[c,d] 60, 72,[c] 80,[g] 88, 92, 93, 94, 95, 96, 107, 114, [g], 115,[g] 116,[g] 118,[g] 119,[g] 120,[g] 121,[g] 122,[g] 123,[g] 124,[g] 127,[g] 134,[g] 135,[g] 136,[g] 137,[g] 138,[g]139,[g] 140,[g] 141,[g] 142,[g] 143,[g] 144,[g] 145,[g] 146,[g] 147,[g] 150,[g] 151,[g] 156,[g] 161,[g] 162,[g] 163,[g] 164,[g] 165,[g] 166,[g] 167,[g] 168,[g] 169,[g] 170,[g] 197 [d]
Others, genital (mucosal)		30,[d] 72,[c] 73,[c] 84,[h] 85, 86,[d] 87, 89, 90, 91, 97, 101, 102, 103, 106, 171, 172, 173

[a]DNA sequence information is available at http://pave.niaid.nih.gov/#explore/reference_genomes/human_genomes
[b]The distinction between frequent and less frequent is arbitrary in many instances. Large descriptive statistics of HPV type distribution by disease are not available for all HPV types. Moreover, many HPV types have been looked for or identified only once or a few times.
[c]First recovered from immunosuppressed or HIV-infected patients.
[d]The malignant potential of this type is definite, probable (e.g., types 26, 53, 66, 68, 73, 82), or uncertain but possible because it has been isolated in one or a few lesions that were malignant (e.g., types 14, 17, 20, 38, and 47).
[e]HPV-46 was found to be a subtype of HPV-20, HPV-64 was found to be a subtype of HPV-34, and HPV-55 was found to be a subtype of HPV-44.
[f]Includes types isolated from a cystic plantar wart, oral cavity, kerathoanacanthoma, hand SCC, or malignant melanoma.
[g]Identified in normal skin.
[h]Identified in normal cervicovaginal cells.

ation is more complex. Species 1, 6, 7, 9, 10, 11, 13, 14, and 15 include only genital (mucosal) HPV types, while species 2 includes only cutaneous types, and species 3, 4, 5, and 8 include both types.

Serotypes

HPV virions induce humoral and cellular immune responses in animals and humans (18, 19). Antibodies react with major (L1) and minor (L2) proteins that constitute the viral capsid. Denatured papillomavirus virions exhibit a common antigen, not displayed during natural infection, which has been used to generate a diagnostic antibody for immunocytochemistry (20). The DNA sequence encoding this common papillomavirus antigen has been mapped to a small region of the gene coding for the major capsid protein. Undenatured virions from different HPV types do not appear to share a common dominant antigen, even though the amino terminus of L2 contains epitopes shared broadly among papillomaviruses (21). Virus-like particles (VLPs) that have the same conformation and size as the native capsid can be made by expressing the capsid genes, either L1 alone or L1 and L2 together. VLPs are the constituents of the current vaccines. The study of papillomavirus L1 VLPs has shown that serotypes generally correspond to genotypes (22).

Composition

Papillomaviruses share the same structure, a naked, T = 7 icosahedral capsid containing a double-stranded, super-helical, circular DNA molecule associated with cellular histone proteins H2a, H2b, H3, and H4. The capsid consists of 72 pentamers that are either pentavalent or hexavalent (Fig. 2) (23). Each pentamer is composed of five major capsid proteins. The minor capsid protein is located in the center of the pentamer, possibly only in the pentavalent pentamers. Purified papillomavirus virions measure 55 to 60 nm in diameter (Fig. 2).

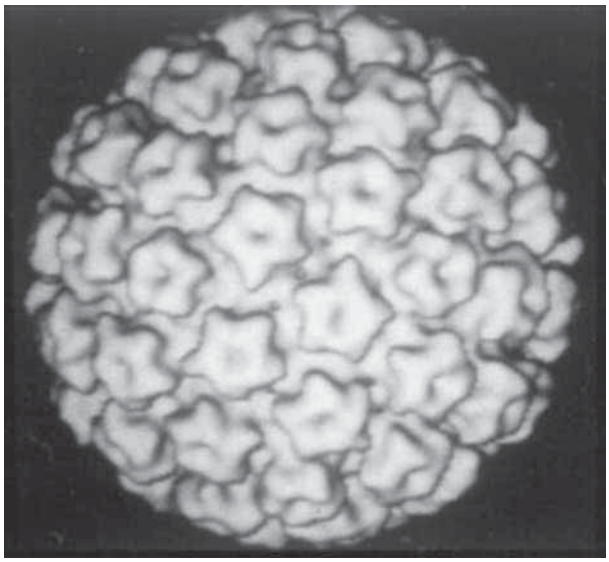

FIGURE 2 Structure of the papillomavirus virion. A cryoelectron micrograph of the HPV-1 capsid is shown (diameter, 60 nm). (Reprinted from reference 11 with permission.)

Genome

The viral genome is approximately 8,000 bp long (40% to 50% G+C content). The apparent molecular weight of 3 to 5 × 106 represents 10% to 13% of the virion's weight. Papillomavirus genomes share the same general organization (Fig. 3), which usually consists of eight ORFs, all located on the same strand (24). These have been designated as either early or late. The early ORFs (E1, E2, E4, E5, E6, and E7), originally numbered by diminishing size, code for nonstructural, regulatory proteins. The E3 ORF does not code for a protein, and different E8 ORFs have been identified only in Kappa- and Xipapillomaviruses—in the latter, E8 has the properties of E5. E5 is absent from the Beta- and Gammapapillomaviruses, along with E4 from the Etapapillomaviruses. E6 is absent from the Xi- and Thetapapillomaviruses, and E7 is absent from the Omicronpapillomaviruses. The late ORFs (L1 and L2) code for the capsid proteins.

An important, typically 1-kb-long, noncoding region, referred to as the upstream regulatory region (URR) or long control region (LCR), lies between the early and late ORFs. The URR includes the origin of replication (ori), the E6/E7 gene promoter (promoter P97 of HPV-16), and the enhancer and silencers. The URR is thought to determine HPV tissue specificity. The function of the URR is governed by at least several of the internal transcriptional regulatory motifs (Fig. 4) (25–28). These cis-acting elements bind to the various cellular and viral proteins that trans-regulate genomic function. At least four components are shared by the URRs of the papillomavirus types examined so far: (i) a polyadenylation signal for late mRNAs at the 5′ end, (ii) E2 protein binding sites, (iii) an E1 binding site associated with the origin of replication, and (iv) a TATA box in the E6 gene promoter. The molecular mechanisms of URR function and regulation are complex (27). Viral gene transcription results in an abundance of mRNA species due to complicated and multiple splicing patterns that reflect a post-transcriptional regulation that is tightly linked to the state of differentiation of the infected cell (29). These splicing patterns also differ among HPV types (Fig. 4). In addition, full or truncated ORFs may appear on different messages (e.g., E2 and E6). Some mRNAs are translated as fusion proteins (E1 and E4), whereas others are polycistronic (E6 and E7).

Proteins

Because of the multiple splicing patterns of viral gene expression, there are more protein products than ORFs. Table 2 summarizes the information on characterized proteins. L1 is called the major capsid protein because it represents 83% of the viral coat and has hemagglutinating activity (30). Experimentally, HPV L1 induces a strong and type-specific neutralizing antibody response (22, 31). L2 is larger and dispensable in the formation of the capsid, but it appears to be important for both entry and egress of the viral genome and for encapsidation of the viral DNA (21). Experimentally, it also generates a weak but cross-specific neutralizing antibody response.

E1 and E2 proteins are involved in viral DNA replication (see "Biology" below) (28, 32–35). E1, the only viral protein with enzymatic activity, has an ATPase that is part of the helicase function of E1 (32). E1 also contributes to the maintenance of the viral episome and is often absent when the viral DNA is integrated. The E2 of HPV-16 possesses transcription regulatory properties and represses the activity of the E6/E7 promoter by binding to the E2 binding sites proximal to the E6 promoter (Fig. 4) (20). In contrast, viral integration disrupts the E2 ORF and allows the free transactivation of the E6/E7 promoter by several cellular transcription factors (29). The differential methylation of the E2 binding sites appears to be important in carcinogenesis (36).

Differential RNA splicing and proteolytic cleavage produce various forms of the most abundant cytoplasmic HPV protein, E4 (actually E1-E4 proteins, because they retain the first five amino acids of E1) (28, 37). With the exception of a conserved leucine-rich motif that appears to be important in the interaction with the cytokeratin networks, the E4 family of proteins shows substantial heterogeneity at the amino acid level among HPV types. Although the role of E4 remains to be fully defined, these proteins form a filamentous cytoplasmic network that co-localizes with the cytokeratin network of intermediate filaments in the lower epithelial layers. E4 proteins form solitary perinuclear structures in the more differentiated layers of the epidermis. E4 may function in coordination of genome amplification, suppression of cell proliferation through a G2-M arrest, and post-transcriptional gene regulation. It is also speculated to play a role in virus release and transmission.

E5 is a small, membrane-bound protein that has weak transforming abilities in high-risk HPVs (28, 35, 38). However, it is unclear how this is accomplished because the protein is undetectable in tissues and the gene is often found to be deleted in cervical carcinomas. E5 interacts with cellular growth factor receptors, including the epidermal growth factor (EGF) receptor and, in the case of HPV-6, also with the erbB2 and platelet growth factor receptors (Fig. 5). Because it associates with an adaptin-like protein, E5 may interfere with the endocytosis and inactivation of these cell receptors. E5 also binds to the endosomal pore-forming protein, a 16-kDa protein with ATPase activity that participates in the endosomal proton pump. The resulting inhibition of endosome acidification increases the half-life of the EGF receptor. EGF activates the phosphokinase C (PKC) pathway of signal transduction, which leads through the mitogen-activated protein (MAP) kinase activation pathway to the activation of the c-jun and c-fos oncogenes.

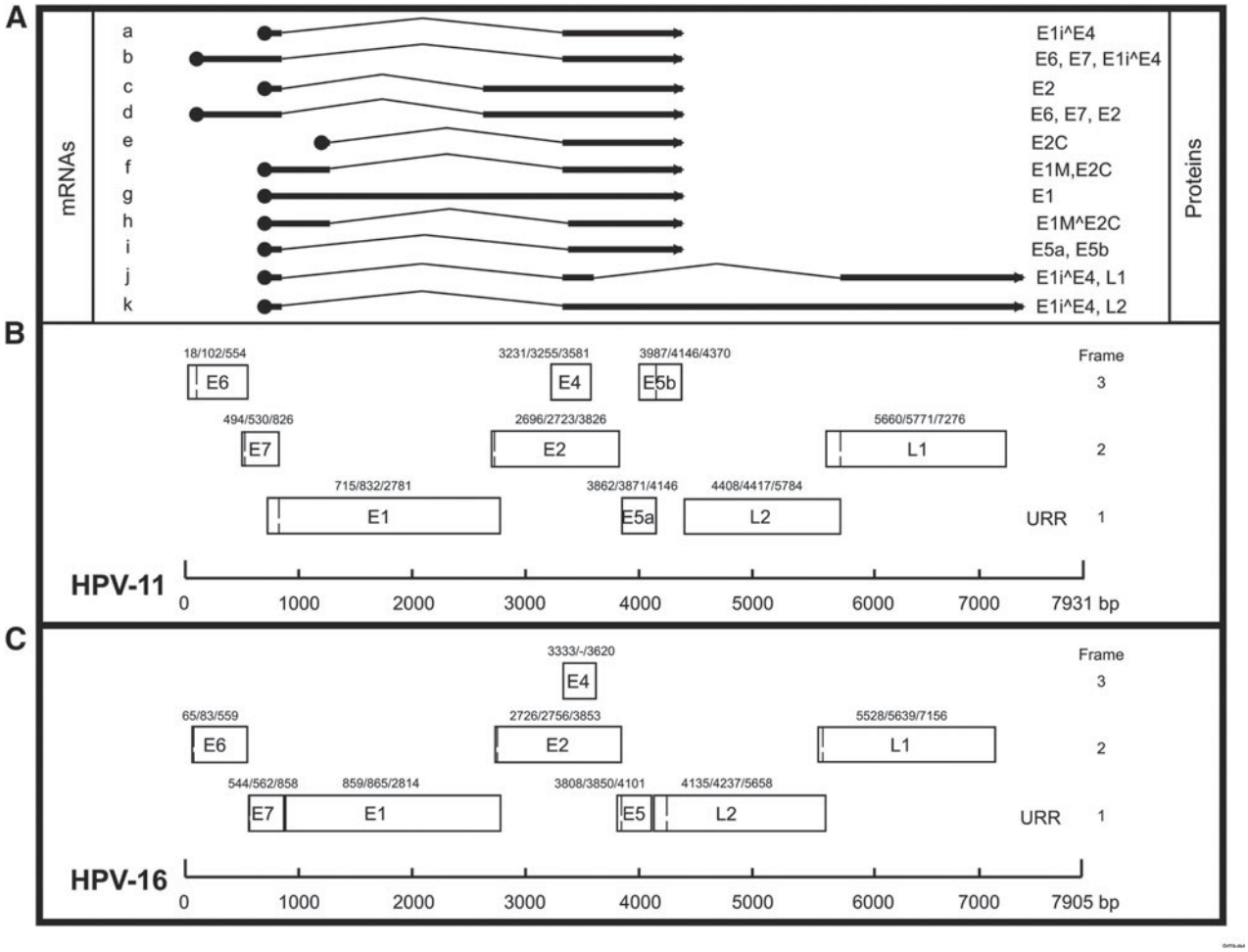

FIGURE 3 HPV genetic maps. (C) linearized HPV-16 DNA map; (B) linearized HPV-11 DNA map; (A) HPV-11 transcription map. By convention, the map origin of papillomaviruses is defined as the position homologous to the HpaI single restriction site of HPV-1. The open boxes correspond to the ORFs in the respective translation frames. The numbers above each ORF indicate the nucleotide position of the preceding stop codon (left solid vertical line)/start codon (dashed vertical line)/stop codon (right vertical line). Each HPV-11 mRNA is depicted with its cap site (solid circle), exons (thick line), introns (thin angled line), and poly(A) site (arrow). The putative corresponding proteins are indicated on the right of the mRNAs.

c-Jun and c-Fos assemble in a heterodimer to form activation protein 1 (AP-1), which has potent transcriptional activity. c-fos is required for the malignant progression of skin tumors. Rather than acidifying the endosome, E5 may act by disturbing the protein trafficking from early to late endosomal structures. E5 may also activate the MAP kinases, ERK1/2, and phospholipase Cγ-1 directly and can enhance endothelin-1-induced keratinocyte growth. E5 may also cause a decreased expression of HLA-A and HLA-B molecules, thus contributing to immune evasion. In addition, E5 suppresses cellular apoptosis and gap-junction communication between keratinocytes.

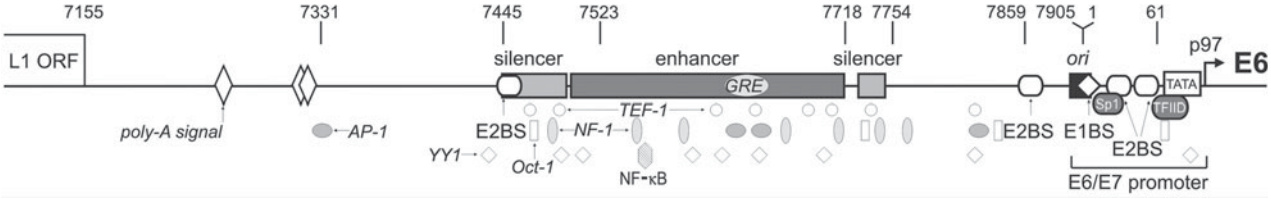

FIGURE 4 Organization of the HPV-16 URR. The URR begins after the stop codon of the L1 ORF and finishes at the cap site of the E6 mRNAs. The positions of some of the potential binding sites of various viral and cellular factors are indicated by the symbols placed on and below the line. E1BS, E1 binding site; E2BS, E2 binding site; ori, origin of replication; TATA, TATA box; GRE, glucocorticoid responsive element. AP-1, Oct-1, NF-1, NF-kB, Sp1, TEF-1, TFIID, and YY1 are cellular transcription factors.

TABLE 2 HPV proteins and their possible functions

Viral protein	Size (kDa)	Functions and properties[a]
E1	68–76	Participation in viral DNA replication
		Has a binding site in the URR and forms a hexameric ring around the DNA (with Hsp 40/70)
		Forms a heterodimer with E2
		Interacts with several of the cellular replicative proteins (DNA polymerase, primase complex, RP-A, p80/Uaf1, etc.)
		Associates with histone H1
		Binds to Ini1/hsSNF5, a chromatin remodeling complex that facilitates the access of transcription factors to DNA regulatory sequences
		Interacts with several cyclin/cyclin-dependent kinase complexes, especially cyclin A/E-Cdk2, leading to E1 phosphorylation, which is necessary for E1 nuclear localization
		DNA-dependent ATPase and helicase activities (with Topo1, E1-BP/TRIP13, p56, Ubc9)
		Nuclear import (with ERK1 and JNK2, importins α3/ α4/ α5, Crm-1 exportin)
		Maintenance of viral episomes (p80/Uaf1)
		Amplification of viral genomes in differentiated cells (with caspases-3/-7)
		Transcriptional repression (alone)/activation (with E2)
E2	40–58	Participation in DNA viral replication
		Has several binding sites in the URR. High-risk HPVs have four binding sites.
		Forms a heterodimer with E1 and dramatically increases the DNA-binding specificity of E1
		Partitioning of viral episomes during cell replication by interacting with Brd4, a double bromodomain-containing chromatin adaptor protein
		Recruits histone acetyltransferases and may alter chromatin remodeling
		Viral transcription repression of E6/E7 at high level
		Viral transcription activation (E2 N-terminus) at low level
		Cellular differentiation transcription regulation, including through interaction with the C/EBP transcription factors (control of MMP-9, IL-10, hTERT, β4-integrin, SF2/ASF)
		Interacts with L2 and may contribute to encapsidation of viral DNA
		Induces apoptosis (estrogen- and progesterone-dependent effect)
E4 (several) and E1-E4	10–17	Most abundant cytoplasmic viral protein, but function poorly defined
		Bind to the cytokeratin filament network
		Are associated with the cornified cell envelope and may contribute to virion assembly, release, and transmission
		? Coordination of genome amplification
		? Mitosis blockade (by binding of Cyclin B/Cdk1 and Cyclin A/Cdk2)
		? Post-transcriptional gene regulation by binding to E4-DBP helicase
E5	10	? Malignant transformation
		Binds and enhances epithelial growth factor receptor signal transduction (HPV-6 but not HPV-16) (which controls Erk1/2, AKT, cyclooxygenase, VEGF, c-Cbl)
		Binds to Golgi's vacuolar ATPase 16-kDa protein (proton pump) and inhibits the acidification of endosomes and Golgi complex, which stabilize the epithelial growth factor receptor and platelet-derived growth factor, respectively
		Activates cellular oncogenes c-*jun* and c-*fos*, whose protein products form the AP-1 transcription factor (may be able to activate AP-1 directly)
		Can enhance endothelin-1-induced keratinocyte growth and target p38 MAP kinase, calpain 3, phospholipase C-γ1, ErbB4, EVER1 and EVER2, ZnT-1, Bap31, calnexin, calpactin 1, karyopherin β3, cyclin A
		Might stimulate arachidonic acid metabolism independent of binding to platelet-derived growth factor
		Inhibits cell-to-cell communication through gap junctions (connexins 26 and 43)
		Inhibits cell motility
		Immune evasion by inhibition of HLA-A and HLA-B cell surface antigens
		Apoptosis inhibition (Fas, Bcl-2, Bax)

(*Continued*)

TABLE 2 HPV proteins and their possible functions (*Continued*)

Viral protein	Size (kDa)	Functions and properties[a]
E6	16–19	Inhibition of keratinocyte differentiation
		Binds to E6-AP, a calcium-binding protein and ubiquitin E3 ligase. Together they bind to and cause the degradation by ubiquitination of the tumor suppressor protein p53 and acceleration of its degradation by the ubiquitination pathway. This leads to the deactivation of the tumor suppressor gene *Notch1* and the activation of the oncogene *erb2* (HPV-16 and HPV-18).
		Leads in the same manner to the degradation of cell proliferation regulatory proteins Bak, multicopy maintenance protein 7, c-Myc, DLG
		Stimulation of malignant transformation
		By binding of E6 and E6-AP to PDZ domain proteins (DLG, MUPP1, MAGGI, and hScribble), leading to transformation by loss of cell polarization, cell signaling, and of cell-to-cell communication
		Cellular immortalization
		By binding and degradation with E6-AP of the NFX cellular repressor of the hTERT (telomerase) promoter, thereby causing elongation and repair of chromosome telomeres
		Stimulation of cell proliferation
		Interacts with p300/CBP (transcription coactivator acetyltransferase), paxillin (focal adhesion protein), IRF-3 (transcription factor), E6TP1 (putative GAP protein), MUPP1 (putative DLG analog), Tyk2, and protein kinase N (high-risk HPVs)
		Interacts with Gps2, a protein that binds to p300/CBP (low- and high-risk HPVs)
		Inhibition of apoptosis
		By binding and degrading Bak, FADD, procaspase 8
		By reducing p53 levels
		Inhibition of the immune response (HPV-16 or -18)
		By binding to IRF-3, but also activates NFκB
		By interacting with Tyk2 and interference with JAK-STAT activation of the IFN-α cascade
		By altering the expression and function of TLR-9
		By inhibiting the IL-8 and IP-10 induction of the IFN-γ response
		By down-regulating E-cadherin, which depletes the epidermis from Langerhans cells
		Cooperation with E7 to counteract the E7-induced increased level of p53
		Contributes to chromosome destabilization (high-risk HPVs)
		Enhances DNA integration and mutagenicity (high-risk HPVs)
		Autophagy and metabolism (mTORC-1)
		Regulation of miRNAs
		Maintenance of viral genomes
		Enhances epithelial invasion
		Binding to Bak, Gps2, E6-AP, and zylin (focal adhesion protein) (low-risk HPVs)
E6*	5–8	Has antiproliferative and antiviral replicative effects
		Inhibits E6-directed degradation of p53
10–14		Stimulation of malignant transformation (high-risk HPVs) and cell proliferation (all HPVs)
		Interferes with pRB, a tumor suppressor gene product, and abrogates pRB's inhibitory effect on cell proliferation by releasing E2F, a family of transcription factors involved with the S phase of the cell cycle (high-risk HPVs)
		Interferes with the p107 and 130 proteins, pRB-like proteins that inhibit cell proliferation
		Inactivates the HDAC protein by binding first to the HDAC complex protein Mi-2β and causes increased levels of E2F. HDAC normally represses gene expression by facilitating chromatin condensation.
		Enhances DNA integration and mutagenicity
		Binds c-Jun and FOXM1c and activates the proliferation-associated transcription factors AP-1 and FOXM1c, respectively
		Inhibits cyclin-dependent kinase inhibitors $p21^{WAF1/CIP1}$ and $p27^{KIP1}$
		Activates cyclins E and A directly, by binding to cyclin E and histone 1 kinase, and indirectly, by activating the Cdc25A phosphatase

(*Continued*)

TABLE 2 HPV proteins and their possible functions (*Continued*)

Viral protein	Size (kDa)	Functions and properties[a]
		Causes increased levels of $p16^{INK4A}$, an inhibitor of cyclin D1-CDK4 and cyclin D1-CDK6. But because the cell cycle is made resistant by E7 to the growth arrest that would otherwise ensue, $p16^{INK4A}$ has been used as a biomarker of potential malignancy
		Causes resistance to TGF-β, an inhibitor of keratinocyte growth
		Causes an increased level of p53 that is counteracted by E6
		Causes centriole amplification that results in aneuploidy
		Apoptosis modulation (estrogen- and progesterone-dependent effect)
		By interacting with p53 and binding to p600 (involved with anchorage-independent growth and cellular transformation)
		By interacting with the I*k*B kinase complex, leading to an increase in the precursor NF-*k*B proteins
		By inhibition of caspase 8
		Inhibition of the immune response (all HPVs)
		By binding and inhibiting IFN-α
		By binding and preventing the translocation to the nucleus of P48/IRF-9, which normally binds to the interferon-specific response element
		By binding and inactivating the IFN-γ-induced transcription factor IRF-1, thereby decreasing the expression of IFN-β, the transporter associated with antigen processing 1 (TAP1), and monocyte chemotactic protein 1
		By altering the expression and function of TLR-9
		Causes resistance to TNF produced by cytotoxic T cells
		Some other known and unknown functions
		Angiogenic properties
		Regulates the expression of the calcium-binding S100P protein and of the mitochondrial ADP/ATP carrier protein
		Interacts with the M2 pyruvate kinase, an enzyme of the carbohydrate metabolism, and activates the acid α-glucosidase
L1	54–58	Major capsid protein
		Assembles into pentamers and although it interacts with L2, it is sufficient to form VLPs
		Contains the dominant, type-specific, neutralizing epitope(s)
L2	63–78	Minor capsid protein
		Cleaved by furin prior to virion cell entry and causes a conformational change to the capsid permitting uptake by secondary receptor (? annexin A2)
		Facilitates vesicular trafficking and the egress of the viral DNA from the endosome after entry
		Interacts with E2
		Facilitates transport of L1 protein to the nucleus and localization to the POD nuclear domains
		Binds to HPV DNA
		Facilitates viral gene transcription
		Stabilizes capsid (stoichiometry unclear)
		N terminus contains minor, cross-reactive, neutralizing epitope(s)

[a]TRIP-13, thyroid hormone receptor interactor 13; VEGF, vascular endothelial growth factor; DLG, disk large; MUPP1, multiple PDZ protein1; IRF-3, interferon regulatory factor 3; TLR-9, Toll-like receptor 9; HDAC, histone deacetylase; mTORC-1, mammalian target of rapamycin complex 1. Note that most of these data are derived from the study of HPV-16.

E6 and E7 are nuclear proteins that play a central role in malignant transformation (28, 35, 39–41). Both proteins bind to a variety of cellular factors (Table 2), with the highest affinity belonging to the E6 and E7 proteins of high-risk oncogenic HPVs, like HPV-16 and -18. Thus, what follows mostly applies to E6 and E7 of the high-risk HPVs.

E6 is a zinc-binding protein that binds tightly to specific motifs on double-stranded DNA (40). It also binds to the E6-associated protein (E6-AP), also known as the E3 ubiquitin ligase. In combination, these two proteins associate with the p53 protein, prompting the accelerated degradation of p53 through the ubiquitin pathway. p53 is an important cell cycle regulator, a tumor suppressor protein, that is activated by DNA damage. Among its different functions, p53 contributes to cell cycle arrest in the G1 phase (Fig. 6). It transactivates the gene families WAF1/CIP1 and INK, whose respective p21 and p16INK4A proteins directly interact with a cyclin/cyclin-dependent kinase/proliferating cell nuclear antigen (PCNA) complex that is responsible for phosphorylating and inactivating the retinoblastoma (pRB) protein. The Notch1 gene is induced; its protein is a determinant of keratinocyte differentiation and acts as a tumor

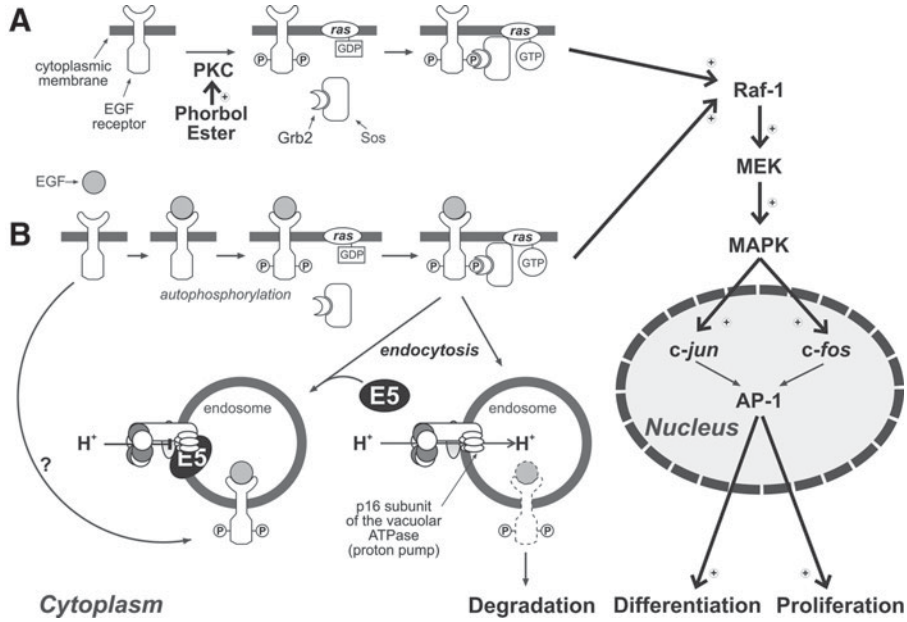

FIGURE 5 Epithelial growth factor (EGF) receptor, phorbol ester, and HPV E5 protein interactions. (A) Upon exposure to a phorbol ester, phosphokinase C (PKC) is activated and phosphorylates the EGF receptor. This allows the binding of a complex of proteins, Grb2 and Sos, that are brought in contact with the cytoplasmic membrane. The Ras protein becomes activated by Sos after exchange of GDP for GTP. This is the trigger for the actuation of a cascade of protein kinases, Raf-1, MEK, MAPK, that ultimately induces the expression of the transcription factor AP-1, which is made up of two proto-oncoproteins, Fos and Jun. The net result is a stimulation of cell proliferation and differentiation. (B) Under physiological conditions, the EGF receptor is activated by the binding of its ligand, EGF. Autophosphorylation ensues, which precipitates the sequence of events detailed in panel A. Eventually, the process stops when the EGF receptor becomes internalized by endocytosis. As the endosome becomes acidic, the EGF receptor is degraded. The acidification of the endosome is dependent on a proton pump that includes a p16 protein subunit. Papillomavirus E5 inactivates the proton pump by binding to the p16 subunit. It is believed that the undegraded EGF receptor is then recycled to the cytoplasmic membrane. The overall effect is an increase of cellular differentiation and proliferation.

suppressor protein. The net result is the arrest of the cell cycle in the G1 phase. Another function of p53 is the induction of apoptosis through the downregulation of the bcl2 gene and the activation of the bax gene. E6 interacts with various other cellular proteins, including tumor suppressors (e.g., disk large [DLG], hScrib, and MAGI), transcription factors (e.g., c-Myc, p300/CBP, interferon regulatory factor 3 [IRF3], TBP, hADA3, p-CAF, and AMF-1), and proteins implicated in cell signaling, cell-to-cell communication, cell polarity, cell proliferation (toll-like receptor 9 [TLR9] and PDZ domain-containing proteins such as DLG, MAGI family, MUPP1, TIP-1/2) (Table 1). The virus escapes immune recognition by downregulating the expression IRF3 and TLR9 (42). E6 can cause cell immortalization by interacting with the promoter complex of the human telomerase (hTERT), a ribonucleoprotein enzyme that maintains the length of chromosomal telomeres, thus preventing cellular senescence. E6* proteins are truncated polypeptides resulting from splicing patterns present only in high-risk HPVs. They inhibit the E6-mediated degradation of p53 and have a negative regulatory effect on cellular and viral replication.

E7 is a zinc-binding protein whose amino terminus has homologies to adenovirus E1A and the large T antigen of polyomaviruses (39). E7 binds to and inactivates the hypophosphorylated pRB, a product of the tumor suppressor gene Rb-1. pRB associates with the transcription factor E2F and its associated protein, DP1 (differentiation-regulated transcription factor 1 protein). This results in the arrest of the cell cycle in the G1 phase. E7 protein can also bind to pRB-related proteins p107 and p130 (these three proteins are called the pRB pocket proteins) and through slightly different mechanisms (Fig. 6) cause the same overall effect, namely the triggering of cell cycle progression. E7 sequesters the histone deacetylases (HDAC), thus triggering the activation of pro-angiogenic genes. In addition, E7 inhibits inhibitors of the cyclin-dependent kinases (p21WAF1/CIP1 and p27KIP1), thus stimulating cell proliferation, interfering with IRF1 and IRF9 thus facilitating the virus immune escape, and the downregulating an apoptosis. E7 also possesses some transcription regulatory activity (it stimulates the AP-1 transcription factor), and it is able to cooperate with v-ras in transforming primary rodent cells.

The transforming and immortalizing properties of the E6 and E7 genes have been demonstrated in various cell lines but also in transgenic animals, in which they induce carcinomas at the site of expression. Considerably less is known about the role of E6 and E7 in the low-risk HPVs (39, 40).

BIOLOGY

Replication Strategy

Papillomavirus DNA replication is governed by the state of differentiation of the keratinocytes in the squamous epithelium (Table 3). First, the HPV virion has to enter the basal keratinocyte (43–47). This process begins with the binding of the viral capsid to the heparin sulfate

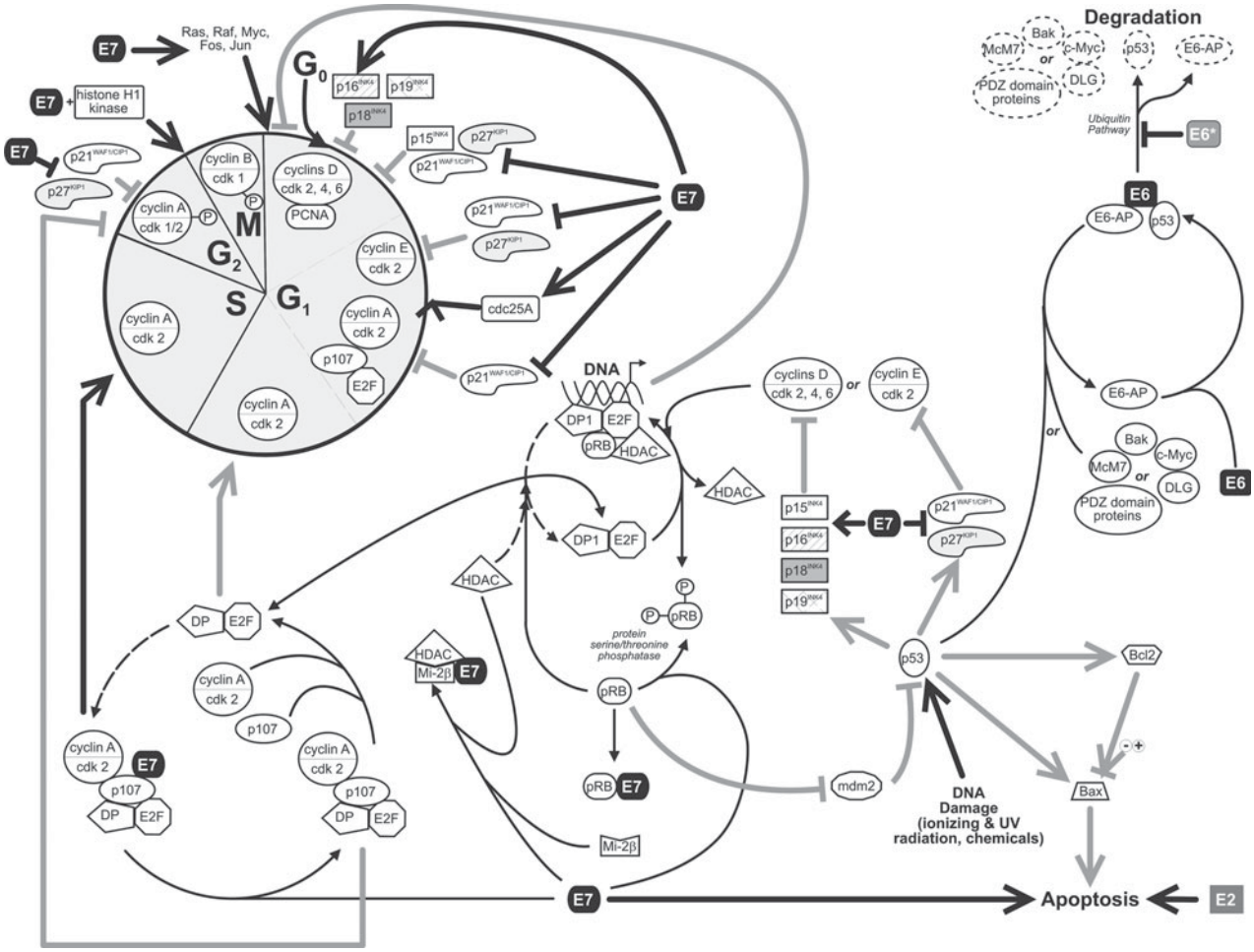

FIGURE 6 Model of the biological interactions of high-risk proteins with the cell cycle and apoptosis (see text for details). Symbols: ρ, activation; σ inhibition. Thick lines with open arrowheads (=J) indicate upregulation; the same lines with a broken end denote downregulation. Thick gray lines represent the regulations in normal cells, whereas the thick black lines show the regulations in HPV-infected cells; thin arrows (=R) show direct interactions. Note that the symbols are not drawn proportional to the protein molecular weights and that the protein complex aggregations are not necessarily concomitant or involve the direct protein-to-protein contacts shown. DP, differentiation-regulated transcription factor polypeptide; HDAC, histone deacetylase; mdm2, murine double minute 2 protein; DLG, disk large.

proteoglycans (various protein backbones covalently "decorated" by heparin sulfate molecules) present in the basal membrane. Syndecan 1 is the most abundant of these proteoglycans. Once bound, the capsid undergoes a conformational change in the vicinity of the basal keratinocyte cell membrane that is mediated by cyclophilin B, a cellular chaperone protein that binds to the L2 viral protein and exposes a furin cleavage site. Once L2 is cleaved by furin, the capsid likely binds a second receptor on the cell membrane. The second receptor has not been firmly identified but could include one or more transmembrane proteins such as integrins and growth factor receptors, which with the assistance of tetraspanin CD151 then allow cellular entry of the viral capsid by endocytosis. Several signaling molecules, including the EGF receptor, PKC, and p21-activated kinase 1 (PAK-1), control this intracytoplasmic process. The viral capsid is fully disassembled in the endosome after acidification. The L2 protein and viral DNA complexes are then transported through the trans-Golgi network with the assistance of retromers, large, multi-subunit protein complexes (45). Eventually, at the time of cell division, when the nuclear envelope is broken, the L2-viral DNA complex can enter the nucleus (47). The viral DNA can then initiate its replication as an episome, generating a low number (less than 20 to 50 copies per cell). Viral replication is dependent on the replicative machinery of the cell and is in part controlled by the transport of E1 into the nucleus, which is dependent on phosphorylation by cellular cyclin-dependent kinases. Viral DNA replication begins with displacement of the histones associated with the viral DNA and the unwinding of the supercoiled viral DNA (34, 48). E1 binds to a receptor in the URR that incorporates the origin of replication. E2 contributes to the specificity of this binding and is then released. E1 has helicase and ATPase activities. In cooperation with E2, topoisomerase I, and replication protein A, E1 displaces the histones associated with the viral DNA and unwinds the DNA supercoiled conformation (Fig. 7). E1 subsequently forms a bidirectional replication fork complex with cellular proteins (polymerase α/primase, DNA polymerase δ/PCNA, replication factor C, topoisomerase II, and DNA ligase). HPV DNA replication progresses bidirectionally from the origin of replication. Viral DNA is then

TABLE 3 Tissue differentiation and HPV markers of productive infection[a]

Tissue layer	DNA	RNA[b]				Virions
		E1, E2	E6, E7	E4, E5	L1, L2	
Stratum corneum	+++					++
Stratum granulosum	+++	++	+	++	+	++
Stratum spinosum						
High	+++	++	++	++	+	+
Low	++	++	++	+		
Stratum basale	+	+				

[a]Relative abundance, from low (+) to high (+++).
[b]In high-grade intraepithelial neoplasias, E6 and E7 mRNAs are usually more abundant than E4, E5, L1, and L2 mRNAs; the converse is true in low-grade lesions. The table applies mostly to anogenital HPVs.

encapsidated in a process that involves its association with cellular histone proteins (H2a, H2b, H3, and H4), but the whole *in vivo* process is poorly understood (49, 50). A transient binding of L2 with E2 protein presumably guides the DNA into the aggregation of viral L1 and L2 proteins that eventually form the capsid. The release of viral particles is probably passive, resulting from the disintegration of the upper squamous epithelium, possibly facilitated by the E1-E4 protein. Desquamating cells are infectious (51).

Host Range

Papillomaviruses are widespread among higher vertebrates, but each type usually has narrow species specificity, and HPVs infect only humans. BPV cause papillomas or fibropapillomas in cattle, but those BPV types producing fibropapillomas (BPV type 1 [BPV-1] and BPV-2) can induce, naturally or experimentally, fibromas and fibrosarcomas in a variety of mammals, such as horses (sarcoid tumor), rabbits, and hamsters (13). The cottontail rabbit (Shope) papillomavirus causes skin papillomas in its natural hosts, the cottontail rabbit (*Sylvilagus floridanus*) and the jackrabbit (*Lepus californicus*), but papillomas and carcinomas in its experimental host, the domestic rabbit (*Oryctolagus cuniculus*) (52). The rabbit oral papillomavirus is a distinct virus that infects the oral cavity of the domestic rabbit and has been used as a model of mucosal infection (53). More recently, a genital murine papillomavirus model of transient infection was identified in *Mus musculus* (54, 55). No animal papillomaviruses have been shown to infect humans.

Growth in Cell Culture and Animal Models

Infection with HPV virions or transfection with HPV DNA of keratinocyte monolayers results in transient replication of the viral DNA without visible cytopathic effect or production of virus. Tissue cultures derived from cutaneous warts or laryngeal papillomas have a normal morphological appearance and no viral gene expression. In contrast, cell lines from cervical carcinomas can exhibit a transformed phenotype and retain HPV DNA, such as HPV-16 (CaSki and SiHa) or HPV-18 (HeLa and C4-1). These cell lines carry HPV DNA in integrated form with a disruption of the early region of the viral genome and therefore do not yield viral particles (permissive infection). Permissive infections have been established using immortalized, nonmalignant cell lines derived from a cervical intraepithelial neoplasia (CIN) containing episomal HPV-16 (W12 cells) or HPV-31b (CIN 612 cells). This has been accomplished by induction of full epithelial differentiation by planting the cells either on the musculofascial layer of the flank of a nude (athymic) mouse (W12 cells) or on an organotypic "raft" culture system (CIN 612 cells) followed by stimulation with phorbol ester, an inducer of phosphokinase C and cell differentiation (Fig. 5). The raft system has undergone further refinements that now enable the propagation of infectious virions in vitro (56, 57).

Immunodeficient mice (e.g., athymic or with severe combined immunodeficiency [SCID]) grafted orthotopically or heterotopically with HPV-infected human xenografts are otherwise the only means to reproduce the natural infectious cycle. They provide sustained propagation of HPV virions and duplicate the macroscopic, microscopic, and molecular features of natural lesions (58). These models demonstrated that HPV-6 and -11 induce condylomata acuminata and that HPV-16 induces intraepithelial neoplasias (59).

Inactivation by Physical and Chemical Agents

Little is known about their resistance to physical and chemical agents, but papillomaviruses are hardy. Viral capsids resist treatment with ether, acid, and heat for 1 h at 50°C. Exposure to 100°C for 1 h is necessary to abolish HPV-11 gene expression in the SCID mouse xenograft model (60). However, HPV is inactivated by autoclaving and 70% ethanol (61). Consequently, standard autoclaving procedures should be used to sterilize surgical instruments that are potentially contaminated with HPV, and the smoke released from vaporized HPV lesions should be evacuated.

EPIDEMIOLOGY

Distribution and Geography

HPV infections and diseases are distributed worldwide, although some geographic variations occur. For example, focal epithelial hyperplasia (Heck's disease), an oral disease related to HPV-13 and -32, mostly affects American Indians, Inuits, and South Africans (62). Geographic disparities in the prevalence of common warts can be seen within countries. The most comprehensive surveys of the worldwide distribution are those of cervical HPV infections and diseases (63–66). HPV DNA is identified in the cervical cells of 11.7% of women with normal cervical cytology worldwide (66). However, this percentage varies from 1.7% of women in Western Asia to 35.4% in the Caribbean. HPV-16 is the dominant genotype worldwide, but there are exceptions such as sub-Saharan Africa where HPV-42 is more common than HPV-16. HPV-18, which is usually second in worldwide prevalence, is replaced at that rank by HPV-58 in South America and by HPV-31, -35, -45, -56, and -58 in sub-Saharan Africa (63).

Overall, in invasive cervical carcinomas (both squamous cell carcinomas and adenocarcinomas), HPV-16 is the most common HPV type (56.6%), followed by HPV-18 (16.0%), HPV-58 (4.7%), HPV-33 (4.6%), HPV-45 (4.5%), HPV-31 (3.8%), HPV-52 (3.4%), HPV-35 (1.7%), HPV-59 (1.3%), and others (65). Although the ranking rarely changes for HPV-16 and -18 according to location, in North America HPV-45, -31, and -33 are, respectively, the third, fourth, and fifth most common HPVs, but in Eastern Asia, HPV-58, -52, and -33 occupy these ranks. The geographic variation is more striking when looking at the variants of HPV-16 with their North American, European, African, Asian, and Asian

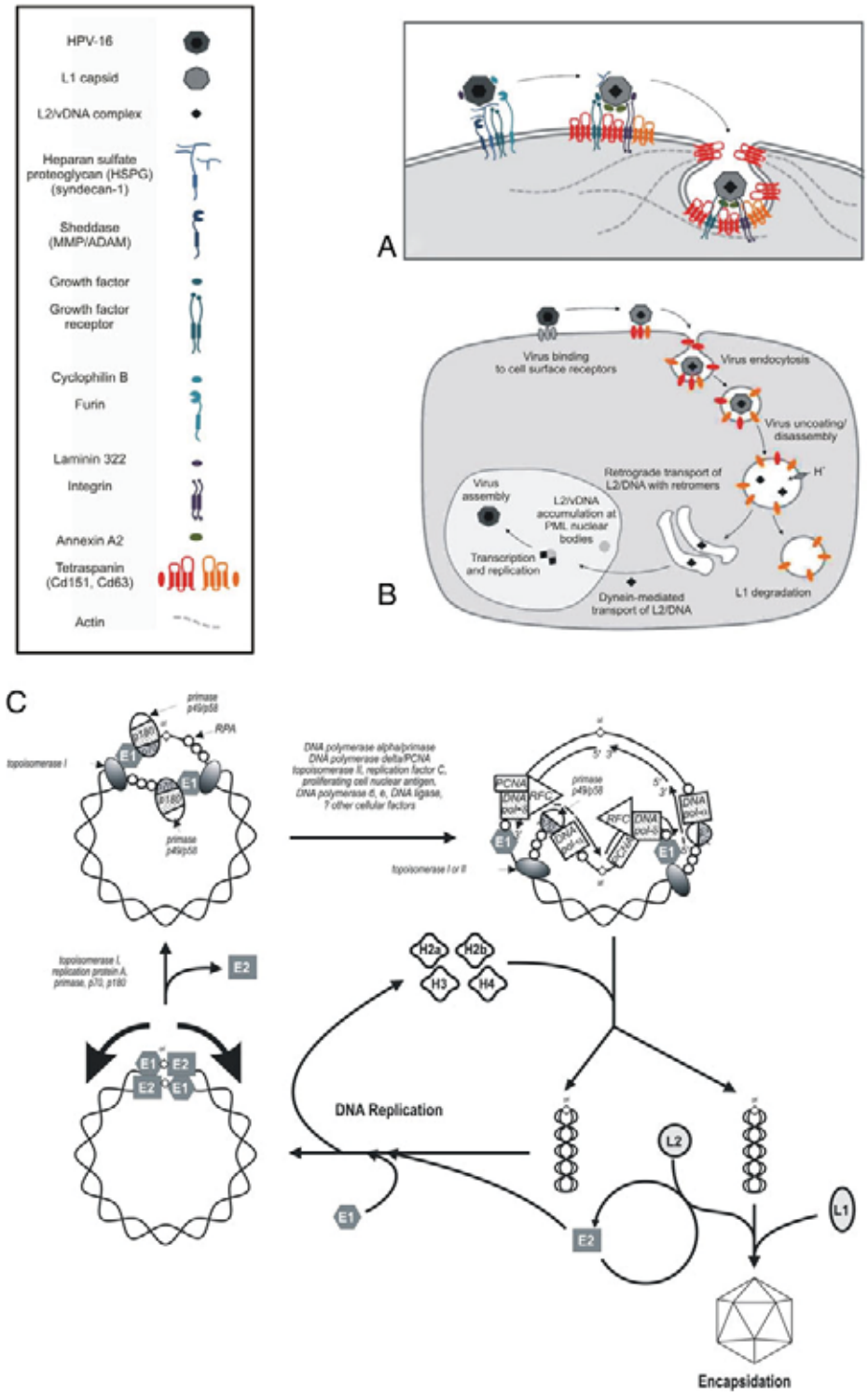

FIGURE 7 Model of HPV entry and DNA replication and encapsidation. (A) shows the cell surface events. Virions bind to heparin sulfate proteoglycans (HSPG) as well as to laminin-332 and growth factors. This induces the activation of growth factor receptors and integrins as well as a change in conformation of the viral capsid, which exposes the amino terminus of L2 for clipping by furin. The HPV particle is then transferred to a second receptor complex that includes tetraspanin before its cellular entry by endocytosis. (B) depicts the intracytoplasmic events with inside the endosome an acidification that leads to viral uncoating. The L2-viral DNA complex is moved through the trans-Golgi network with the help of retromer complexes. It is finally moved inside the nucleus where it accumulates at the PML nuclear bodies. (C) is a schematic rendition of the viral DNA replication and encapsidation, the latter being assisted by E2. Histones proteins (H2a, H2b, H3, and H4) are also incorporated. The virions are released with the death of the keratinocyte, terminally differentiated in a cornified shell. (Figures 7A and 7B are slightly modified from reference 33, with permission.)

American lineages (67). Squamous cell carcinomas represent about 90% of all cervical cancers associated with HPV, and genotypes 16 and 18 are present in 59% and 13%, respectively, of these tumors worldwide. In contrast, cervical adenocarcinomas, which represent the other 10%, contain HPV-16 and -18 in about equal proportions, 36% (65).

Incidence and Prevalence of Infection

HPV infections are endemic. No epidemic patterns of HPV infection have been recognized. The moderate transmissibility, variable incubation period, latency, and spontaneous resolution of HPV infections impede the recognition of HPV disease outbreaks.

Cutaneous Warts

Cutaneous HPV infections are extremely prevalent, with rates of 80% in immunocompetent hosts and 95% in immunocompromised patients (68). In a study of 744 cutaneous warts from 246 patients, the HPV genotypes were HPV-27 (23.7%), HPV-57 (21.8%), HPV-2 (21.7%), HPV-1 (18%), HPV-4 (5%), and others (69). HPV-1 was the predominant genotype in plantar warts of children younger than 12 years old. HPV DNA can be found on healthy skin, especially from types usually associated with epidermodysplasia verruciformis and can still be present in half of the subjects when new samples are obtained from them 6 years later (70, 71)

Cutaneous warts (common, plantar, and flat warts) are very common among the general population. Mostly a disease of school-age children, common (hand) warts represent about 70% of all cutaneous warts (72). The prevalence of common warts ranges from 0.8 to 22% (72). Plantar warts make up to a third of cutaneous warts and are found in a wider age group, primarily adolescents and young adults. Flat or juvenile warts constitute 4% to 8% of the cutaneous warts, with peak prevalence among 10- to 12-year-olds (72). An increased incidence of plantar warts has been noted in the winter months.

Genital HPV Infections and Diseases

No seasonality is recognized in the acquisition of HPV infection or the expression of HPV diseases. In the U.S. female population, one study of urine samples collected from 3,262 sexually active women aged 18 to 25 found that 26.9% of subjects were positive for a genital HPV, with 20% of the total sample being positive for a high-risk HPV (73). Remarkably, 62% of the infections were by multiple types; 14.3% of women reporting only one lifetime sexual partner were positive. A second survey with a sample of 4,150 females aged 14 to 59 years found that between 2003–2006, before the introduction of HPV vaccination, the overall prevalence of HPV DNA in vaginal swabs was 42.5%, with the majority represented by high-risk HPV infections (74). The peak rate was in the 20- to 24-year-old group (53.8%), followed by the 14- to 19-year-old group (32.9%). Here again, having only one lifetime sexual partner offered limited protection, as 18.2% of these women were positive. When repeated over the 2007–2010 period after the introduction of HPV vaccination, overall HPV prevalence declined modestly to 39.8% (75). A significant reduction was noted in the 14- to 19-year-old group (26.1%) but not in the 20- to 24-year-old group (59.8%); the former group was more likely to have been immunized. A meta-analysis of 78 studies of women with normal cytology indicated an overall worldwide HPV prevalence of 10.4%, with prevalence as low as 8% for Asia to as high as 22% for Africa (76). Rates of HPV DNA prevalence on the male genitalia vary from 1.3% to 79%, with a majority of studies reporting rates of 20% and higher (77). In the United States, about 20 million people are estimated to be infected with genital HPVs, half of them aged between 15 and 24 years (78). It is estimated that of the U.S. sexually active population 85% of women and 91% of men will get a genital HPV infection during their lifetime (79).

Most infections are rapidly cleared, or at least become undetectable (80). In a prospective study of 331 18- to 35-year-old women who had HPV DNA-positive cervical samples, half had cleared the virus by 9 months (81). The median time to clearance was longer for high-risk types (9.8 months) than for low-risk types (4.3 months). By 14 months, only a tenth of the subjects were still positive. Typically, with repeated testing over time, only a minority of patients will be positive every time, while another subgroup will be consistently negative (82).

Until recently, the incidence of anogenital warts was increasing in the United States. The number of initial visits for genital warts to physicians' offices in the United States increased from 222,000 in 2000 to its zenith of 453,000 in 2011 but declined to 404,000 in 2013 (83). Wherever the quadrivalent HPV vaccine has been introduced in the world, a major impact commensurate to the rate of vaccine coverage has been noted on the prevalence or incidence of genital warts (84, 85) (see "Active Immunization"). The NHANES survey indicates that in the United States, 5.6% of persons aged 18 to 59 years reported ever having genital warts, and there were more in women (7.2%) than in men (3.4%) (86). A random survey of 69,147 women 18 to 45 years of age from the general populations in Denmark, Iceland, Norway, and Sweden showed that 10.6% reported ever having had clinically diagnosed genital warts (73). The peak incidence is around ages 25-29 years in males and 20–24 years in females (74).

The prevalence of HPV infections in neonates and infants appears to be low (87). No more than 1% of babies have oral or genital HPV infections. Genital warts are highly unusual in children (88). Fifty percent to 75% of children with genital warts acquire them from sexual abuse, and 1 to 2% of sexually abused children have genital warts (87). Nevertheless, the presence of genital warts alone is not sufficient to establish a case of sexual abuse. Nonsexual transmission of anogenital warts appears to be possible (87, 89, 90). Family members of children with anogenital warts have been shown to have cutaneous warts that harbor HPV-6. Conversely, the presence of HPV-1 or HPV-2, nongenital genotypes, in about 20% of genital warts recovered from prepubertal children suggests the existence of nongenital sources, such as autoinoculation or nonsexual contact but does not necessarily exclude fondling (87). In young children, anogenital warts are more likely the result of vertical transmission, whereas horizontal nongenital or genital transmission is more likely in older children, but there is no firm age cut-off (90, 91). Recommendations for the evaluation of children with genital warts suspected of sexual abuse are available (92).

The prevalence of cervical HPV disease is best measured by cervical cytology. Rates vary between 0.9 and 4.8% depending on the criteria and age and decrease after age 24 (93). In one study, 2.5% of cervical cytology samples had evidence of HPV-related cervical disease with low-grade SIL (LSIL) (1.97%), high-grade SIL (HSIL) (0.51%), SCC (0.026%), and adenocarcinomas (0.0046%) (94). In addition to these well-recognized entities, ambiguous cytological abnormalities are as common. They are called atypical squamous cells (ASC), either of unknown significance

(ASC-US) or which cannot exclude HSIL (ASC-H) (both subcategories used to be grouped as ASCUS) (95).

An HPV infection is present in virtually all cervical cancers (SCC and adenocarcinomas) (96, 97). It is a necessary, if not sufficient, condition for the development of cervical cancer (86). In 2008, cervical cancer was the third most common cancer in women worldwide, with 529,800 new cases and 275,100 deaths (98). The burden is mostly in developing countries, where 86% of the cases occur. In the United States, according to 2014 estimates, cervical cancer is in decline; it ranks 14th in incidence (12,360 new cases) and 16th in mortality (4,020 deaths) among all female cancers (99).

Cervical cancer is not the only cancer attributable to HPV. In 2008 global estimates indicated that 100% cases of 530,000 cervical cancers could be attributed to HPV worldwide, but so could 43% of 27,000 vulvar cancers, 88% of 27,000 anal cancers, 50% of 22,000 penile cancers, 70% of 13,00 vaginal cancers, and 27% of 85,000 oropharyngeal cancers (100). Globally, 16.1% of new cancers are attributed to an infection, with about 30% of that fraction caused by HPV worldwide (101). These figures do not account for the premalignant conditions attributable to HPV.

Recurrent Respiratory Papillomatosis

The age of onset of recurrent respiratory papillomatosis has a bimodal distribution that includes young children and young adults but not the elderly (102, 103). In the United States, the estimated annual incidence rates are 1.7-4.3/100,000 in children less than 14 years of age and 1.8/100,000 in young adolescents (older than 15 years) and adults. The estimated prevalence rates for these two populations are 10.9/100,000 and 4.5/100,000, respectively.

Other HPV Infections and Diseases

HPV can be present in the oral cavity (104). Among the U.S. population, 7.3% of individuals were estimated to have one or more oral HPV found in an oral rinse (105). However, only 1.9% of young female enrolled in an HPV vaccination study were found to have the presence of oral HPV DNA (106). Focal epithelial hyperplasia (Heck's disease) is also a relatively uncommon disease that is mostly found in children and adolescents (62). Epidemiological data are lacking for other HPV diseases, such as the very rare epidermodysplasia verruciformis.

RISK FACTORS

Cutaneous Warts

Use of heated swimming pools or communal baths is an activity that appears to promote the acquisition of plantar warts, and in one controlled experiment, students who wore protective footwear during swimming were much less prone to developing plantar warts than those who did not (107–109). However, a large study has failed to show an increased transmission rate in public areas (110). Transmission within the family home may be the most common mode of acquisition (111). Nail biting is associated with the presence of periungual warts.

Some primary (see "Immune Responses" below) and secondary immunodeficiencies are risk factors for acquiring cutaneous warts. An approximate 10-fold increase in prevalence of cutaneous warts has been noted in lymphoma patients, whereas a 4- to 12-fold increase has been reported for human immunodeficiency virus (HIV)-infected individuals (112). Organ transplant recipients are particularly susceptible to developing cutaneous warts or HPV-containing SCC in the sunlight-exposed areas of their body (112). Three years post-transplantation, between 25 and 42% of patients have cutaneous warts; this number rises to 92% after 5 years or more (112). In transplant patients the lifetime risk of developing SCC or basal cell carcinoma increases 100- and 10-fold, respectively (112, 113).

People working with meat, poultry, or fish are uniquely susceptible to hand warts and up to one-half of them are affected (103). Particularly intriguing is the predominance of HPV-7 in these lesions, since HPV-7 has rarely been found elsewhere, with the exception of oral lesions in immunosuppressed or immunodeficient patients. Associations between UV tanning beds and body warts and between cocaine snorting and nasal warts have also been reported.

Genital HPV Infections and Diseases

Sexual intercourse with a partner who has genital warts is a risk factor for the development of genital warts. About two-thirds of the sexual partners of persons with anogenital warts will develop the disease within 2 years (93, 114). The risk of developing genital warts is most strongly and directly linked to lifetime number of sexual partners (93, 115). Other markers of sexual behavior, such as sexually active years and number of regular partners, are additional risk factors. Markers of sexual behavior yet to be identified could be the confounding variables explaining inconsistent risk factors such as cigarette smoking, contraceptive use, prior sexually transmitted diseases, pregnancy, and alcohol consumption (93, 115).

Consistent with sexual transmission, age is a major risk factor for the acquisition of cervical infection, with a peak in the 14- to 19-year-old group (116). Genital HPV infection is very uncommon in virgins, but when present it is likely the reflection of nonintromissive sexual play (117). Such activity, practiced by about a third of self-declared virgin adolescents, is not regarded by the subject as sexual intercourse, yet it is conducive to the transmission of infectious agents, including HPV. The risk of infection rapidly decreases after age 30, but an unexplained second peak at older ages may occur (80). Sexual behaviors begun at an early age, higher number of lifetime sexual partners, and higher number of recent sexual partners are risk factors (80). Lesbians are not free from these risk factors (118). Sexual partners share, at least in part, the same HPV infections or diseases (76,119–124). Similarly, the association between anal warts and anal intercourse also indicates a direct route of transmission through sexual contact (76, 114). The past and present sexual activity of the male partner is also important (125). Less consistent risk factors include the use of oral contraceptives, smoking, not using condoms, and some HLA polymorphisms (80, 126, 127). For example, HLA class I allele A*0301 increases the risk of cervical cancer, while B*1501 decreases it (128) (see the "Pathogenesis in Humans" section).

There may also be a protective effect from the consumption of fruits and vegetables or from absorbing or having circulating levels of vitamins C and E, carotenoids, and xanthophylls (80). Other potential risk factors include multiparity, other sexually transmitted infections (herpes simplex virus and Chlamydia trachomatis), and cervical chronic inflammation. The risk factors may have different effects on low-risk and high-risk HPV infections and how they contribute to the progression from infection to premalignancy and to cancer (80, 129). In that regard, the most

decisive factor is the HPV genotype, and HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59 are recognized carcinogens (130–132). Different HPV types have different oncogenic risks, and HPV-16 is very clearly the most oncogenic of all (133). Different variants of HPV-16 carry their own oncogenic profiles, with the African and Asian variants being more oncogenic than the European ones (67). Other HPV types are probable, HPV-68, or possible carcinogens, HPV-26, -53, -66, -67, -68, -70, -73, and -82 (132). Multiple HPV infections are common, especially in CIN lesions, and there is a clustering of some HPV types with one another, although the significance remains unknown (134).Very similar risk factors for anogenital HPV infections have been implicated in males, including number of sexual partners, lack of condom use, smoking, and lack of circumcision (135).

Although less well studied, the risk factors related to sexual behavior, sexual partner history, and presence of high-risk oncogenic HPV DNA have been identified in the development of anal, penile, and vulvar or vaginal SCC (100, 115). Anal receptive intercourse, in both men and women, is a risk factor for the acquisition of anal warts and preinvasive and invasive SCC (136). Because all genital HPVs share the same mode of transmission, it is not surprising that a history of anogenital warts carries an increased risk for the presence of all of these anogenital cancers (115).

Renal allograft recipients have a 10- to 100-fold increased risk of developing anogenital warts, intraepithelial neoplasias, and cancer, a risk that is proportional to the duration of immunosuppression (113). The risk probably extends to other allograft transplant recipients and has been observed in women treated with immunosuppressive agents for glomerulonephritis. Cervical and oral malignancies associated with HPV have also been observed in these populations (137–139). Patients with diabetes mellitus may be at risk for the development of condyloma acuminatum. However, diabetic women do not appear to have an increased risk of cervicovaginal infection during pregnancy. Furthermore, general immune responsiveness appears to be blunted in older women with HPV infection compared to age-matched controls (140).

The association between infections with HIV and anogenital HPV has been well documented. Conversely, the presence of genital HPV infection appears to about double the risk of HIV infection acquisition (141). In HIV-seropositive women, depending on populations and methods of diagnosis, there is a 2- to 40-fold increase in the incidence and prevalence of condylomata acuminata and CIN compared to that in HIV-seronegative controls (142–146). This added risk is present even in HIV patients with a normal CD4 cell count, although it rises with lower CD4 counts or higher HIV viral load. HIV infection probably promotes the reactivation of HPV infection rather than new acquisition, since HIV-seropositive women shed HPV more persistently and in larger quantities than controls do (125). The quantity and persistence of HPV DNA, as well as the presence of high-risk HPV types within lesions, are risk factors for the development of CIN (147, 148). HIV infection also increases the incidence of vulvar (VIN) and vaginal (VAIN) intraepithelial neoplasias, a risk also augmented with a low CD4 cell count (<200 cells/ml) (145, 149). Anal intraepithelial neoplasias (AIN) are more common in HIV-positive men and women (150–152). This risk and that of high-risk anal infection is greater in men who have sex with men (MSM) than in heterosexual men, 32% versus 7%, respectively (151, 153). Curiously, MSM do not have a higher risk of penile HPV infection than heterosexual men, both having an approximate 8.5% prevalence and similar incidences (153). In addition to HIV infection and receptive anal intercourse, the factors that increase the risk of AIN include a low current or nadir CD4+ cell count, high-risk HPV type infection, persistent anal HPV infection, multiple HPV type infections and high anal viral load (154). Concomitant anal and cervical infections or lesions are more common in HIV-seropositive than in seronegative women (155). Furthermore, the HPV genotypes are more likely to be concordant in the two anatomic sites if the subject is HIV-seropositive than seronegative, 18% versus 3%, respectively (155). Surprisingly, HPV has been found to be more prevalent in the anus (79%) than in the cervix (53%) in HIV-seropositive women (156). The same imbalance is found in the anus (43%) and cervix (24%) in HIV-seronegative women (156). The risk for oral warts and HPV-associated cancers is likely elevated in HIV-infected persons (157).

Cervical cancer was incorporated in the case definition of AIDS in 1993 (158), but it was later noted that the risk of invasive cervical cancer did not increase with increasing time after AIDS or correlate with the CD4+ cell count at the time of cancer diagnosis (159). In other words, although HIV infection was associated with an increased risk of cervical cancer (as well as of cancers of the anus, vagina, vulva, penis, and oropharynx), this risk and that of anal cancer did not appear to be related to HIV-induced immunosuppression (160, 161). Nevertheless, more recent studies indicate HIV-related immunosuppression contributes to the risk of cervical cancer (162–164). This has not been established with anal cancer, although the increased risk for this cancer in solid organ transplant recipients argues in favor of an immunosuppressive effect (165, 166). Undoubtedly, different confounding factors are at play, because the HIV infection is a risk factor for anal cancer in males but not in females (151). One of these factors is the recent availability in some areas of anal cancer screening, which likely contributes significantly by early detection to the very rapid rise in the incidence of anal cancer *in situ*, something that has not otherwise altered the steady but much slower rise in invasive anal squamous cell carcinoma incidence (167).

Recurrent Respiratory Papillomatosis

The juvenile- and adult-onset variants of recurrent respiratory papillomatosis are usually linked to different risk factors (102, 103). With the juvenile-onset form, the children are often firstborn and delivered vaginally by teenage mothers who have condyloma acuminatum. For the adult-onset form of the disease, patients report a higher number of lifetime sexual partners and a higher frequency of oral sex than controls. The disease is very rare among siblings. Unexpectedly, recurrent respiratory papillomatosis has not been reported to occur in transplant recipients or HIV-infected individuals.

Association between HPV and Cancer

HPV infection is a necessary condition for the development of preinvasive and invasive cervical cancer (66, 168, 169). The epidemiological evidence can be summarized as follows: (i) HPV DNA is consistently present in virtually all CIN, SCC, or adenocarcinoma specimens, and specific genotypes (mostly 16 and 18), so-called high risk, are responsible for this association; (ii) the presence of HPV-16 antibodies is associated with cervical cancer (18, 170); (iii) the prevalence of the high-risk HPV types increases relative to the low-risk types as the grade of the preinvasive cancer worsens (64); (iv) the strength of the association between HPV and

preinvasive or invasive cervical cancers, as measured by the relative risk, ranges in most studies from 20 to 70, which is larger than the magnitude of the association between lung cancer and smoking (64); (v) no other risk factors for preinvasive and invasive cancers approach the strength and consistency of HPV infection (64); (vi) persistent but not transient exposure to high-risk HPV leads to cervical disease progression, which suggests that the cancer risk disappears with exposure cessation (80); and finally, (vii) HPV infection precedes the development of preinvasive cancer.

High-risk HPVs are also implicated in the development of SCC at sites other than the cervix (66, 168, 169). It is estimated that 88% of anal cancers, 70% of vaginal cancers, 50% of penile cancers, 43% of vulvar cancers, and 27% of oropharyngeal cancers are attributable to HPV (100). In the case of the penile, vulvar, and vaginal cancers, the percentage attributed to HPV is not higher because among those cancers, only the basaloid and Bowenoid histologic variants are related to HPV.

In the nasal and sinus cavities, four of five exophytic papillomas and only a third of the inverted (Schneiderian) papillomas contain HPV, usually HPV-6 or HPV-11 (171). HPV likely plays a role in some of the SCC of the mouth, sinonasal tract, and larynx (168, 172-175). However, strong evidence has now emerged to attribute up to 70% of oropharyngeal cancers to oncogenic HPVs, mostly HPV-16 (175). The incidence of these HPV-related oropharyngeal cancers is rising (176). They tend to occur in younger subjects and are associated with sexual behavior markers of young age at intercourse, number of lifetime sexual partners, history of genital warts, performance of oral sex, and number of oral sex partners (174, 175). Limited or conflicting evidence exists for an association between HPV infection and SCC of the esophagus, lung, breast, colon, urothelium, prostate, ovary, and endometrium (168, 173,177–181). Squamous cell carcinomas associated with HPV-16 of the conjunctiva (possibly also prompted by ultraviolet light exposure or HIV infection) and around the nail bed have been described (182–184).

Nonmelanoma skin cancers (NMSCs) include mostly SCC and basal cell carcinomas. Actinic keratoses represent the precursor lesion for SCC. HPV infection is present in 33 to 99% of NMSCs and precursors in immunosuppressed patients and in 21 to 100% of immunocompetent patients (185–187). A wide assortment of cutaneous and anogenital HPV types are detected in these NMSCs (188). However, the great majority of the genotypes are related to epidermodysplasia verruciformis-associated HPVs, for which better understanding of their oncogenicity is needed (189, 190). The clear oncogenic potential of a subset of these HPVs may, along with UV light, contribute to the development of NMSCs and precursor lesions. However, a causal link with HPV is difficult to prove for several reasons: (i) the high number of HPV types, including most of the novel ones, that are isolated from these lesions is perplexing; (ii) the HPV types found vary from patient to patient but also from lesion to lesion, and multiple infections are common; (iii) these HPVs are found in the normal skin (and in a small number of melanomas) of 33% to 73% of tested subjects; (iv) they are typically present at low copy numbers; and (v) they are often at the surface of the epithelium rather than within (185).

Reinfections

HPV-associated diseases can recur, and relapses are well documented. How often these events represent reinfection versus relapse of a previous subclinical or clinical infection is often impossible to determine. Laryngeal and genital HPV infections can remain latent and subsequently relapse either at the site of the former lesion or in the normal-appearing skin surrounding the ablated lesion (191, 192). Five types of observations suggest that relapses are more common than reinfections. First, attempts to treat the male sexual partners of women with genital HPV lesions have had no effect on the treatment failure rate in the females (193). Second, studies of HPV types present in the lesions of both sexual partners show only partial concordance either at the genotype or subtype level (76, 119–124). In males but not in females, a history of genital warts in the previous 5 years in the sexual partner does not predict anogenital wart recurrence. Third, the frequent presence of HPV-6 or HPV-11 DNA in the plucked pubic or perianal hairs of patients with condyloma acuminatum indicates a potential viral reservoir for relapse (194). Fourth, with the use of ever more sensitive and specific HPV PCR assays, it is possible to demonstrate that infections that appear to be transient are in fact persistent (195, 196). Fifth, therapeutically induced immunosuppression in solid-organ transplantation leads to an increase of HPV infections and diseases (see "Risk Factors" above). Although this could be the result of increased susceptibility to HPV, relapse of infection is a more likely explanation.

Several studies on the male condom detailed in the Prevention section indicate that reinfection may also contribute to recurrences. Moreover, recurrence of genital warts affects subjects who do not use condoms or involves anatomical areas not protected by the male condom (197).

Transmission

Routes

The epidemiology of plantar warts suggests an indirect mode of transmission, for example, through fomites present in communal baths or swimming pools (109, 110). The virus probably enters the epidermis directly, because special footwear is protective. Direct natural or experimental transmission is possible (198), as is autoinoculation from genital lesions. Because epidermodysplasia verruciformis is so rare, until recently it was unclear where the natural viral reservoir was. It is now evident that these HPV types are widely distributed at low levels in the population, in normal skin, or in actinic keratoses and NMSCs (185, 188).

As discussed above, young children can acquire anogenital HPV infections by nonsexual routes, like *in utero* and perinatal exposure, nonsexual direct contact, and autoinoculation. However, the importance of alternate routes of transmission is probably negligible. Even the very low rate of HPV infection in virgin women does not exclude all forms of sexual activity (117). Although HPV DNA can be recovered from the underwear of women with anogenital warts or on surgical instruments used for gynecologic examination, a role for fomites in the transmission of anogenital HPV has not been demonstrated, but it is reasonable to assume that sex toys could be such a vehicle.

There is evidence that oral HPV infections are transmitted sexually and that there a concordance between the types found in the genital area and the oral cavity (105, 106).

The mode of transmission of respiratory papillomatosis has not been directly established. In its juvenile-onset form, the disease could be acquired by vaginal delivery through an HPV-infected birth canal, whereas the adult form of the disease might result from oral sex (103). Nevertheless, respiratory papillomatosis can be present at birth or after ce-

sarean delivery, indicating possible *in utero* transmission (103). Transmission among family members is very rare.

Risk Factors

The risk of transmission after direct or indirect contact with cutaneous warts is probably low and may be mitigated by the presence of cofactors such as the immunity of the recipient and the nature and duration of the contact (196). Plantar warts tend to appear on weight-bearing areas. This suggests that microscopic injury to the skin fosters viral entry. Shaving (even using depilatory creams), finger sucking, or nail biting may favor the transmission or extension of lesions and cause a Koebner phenomenon (lesions appearing on a line of trauma) (198–202). In vitro observations suggest that the wound repair epithelium is more susceptible to an active infection. However, manual workers other than meat packers, abattoir workers, or fish handlers do not appear to be prone to the development of warts, and trauma was not found to be a risk factor in meat handlers. Perhaps moisture and warmth are additional risk factors (109).

The risk of developing condyloma acuminatum or acquiring an anogenital HPV infection after one sexual contact with an affected individual is unknown but is presumably low, but as expected, repetitive contact increases the risk. In a study of 603 female college students negative for HPV DNA at baseline, resampling every 4 months with a cervicovaginal lavage found a cumulative prevalence of 29% at 1 year, 32% at 2 years, 48% at 3 years, and 54% at 4 years (117). In a study of 18- to 20-year-old male students sampled at 4-month intervals, the cumulative incidence of any genital HPV infection was 62% after 2 years (203). About two-thirds of sexual partners of individuals with anogenital warts will develop warts within 2 years (114). Genital warts appear in areas, such as the vulvar fourchette, that are more likely to be traumatized during intercourse; microabrasions or lacerations are also thought to play a role in HPV transmission.

Macroscopic lesions are possibly more contagious than microscopic ones. In one study of women with HPV infection, 67% of 42 male consorts of women with visible condylomata acuminata developed clinical or subclinical lesions, compared to 46% of 39 partners of women with subclinical lesions (204). Transmission of condyloma acuminatum by household contact is probably very rare in the absence of sexual contact or abuse.

The risk of acquiring recurrent respiratory papillomatosis following vaginal delivery from a woman with anogenital warts has been estimated to range between 1:80 and 1:500 (102, 103). Firstborn babies carry more risk than babies of subsequent births, presumably because of longer delivery (103). Furthermore, viral load has been shown to condition the risk of HPV-16 transmission from mother to child. Oral sex appears to be a risk factor for the adult-onset form of the disease.

Nosocomial transmission is addressed in the "Prevention" section.

Duration of Infectivity

The duration of infectivity is unknown. Patients with anogenital warts of long duration may be less infectious than those with a shorter disease history (114).

PATHOGENESIS IN HUMANS

The incubation period for HPV diseases is poorly understood. For cutaneous warts, experimental inoculation leads to lesion development within 3 to 4 months on average, but as early as 6 weeks or as late as 2 years (198). Similar incubation periods have been observed in sexual partners of patients with condyloma acuminatum for whom the date of exposure could be estimated (114, 205). In a study of 51 female university students with an incident cervical HPV-6 or -11 infection, 51% developed genital warts within a median of 2.9 months (112). Among 603 subjects monitored over a 36-month period after an incident HPV infection, 47% and 29% of the students developed LSIL of the cervix and vagina, respectively (117). The median times for lesion clearance were 5.5 and 4.7 months, respectively. When limited to HPV-16 and -18 infections, the cumulative incidences for CIN 2 and CIN 3 were 20% and 7%, respectively. Half of cutaneous warts disappear within 1 year (206, 207).

Virus Replication

HPVs infect squamous keratinized (skin) and nonkeratinized (mucosal) stratified epithelia. The mucosae of the mouth, upper airways, vagina, cervix, and anal canal are major targets, but HPV has been found in other locations such as the conjunctiva, lacrimal sac, nasal passages, bronchi, esophagus, cervical glandular tissue, and bladder (171, 208–211). Epithelia next to a squamocolumnar junction (sinuses, larynx, anus, and cervix) are particularly vulnerable. In the uterine cervix, a process called squamous metaplasia triggered by hormonal and pH changes causes this squamocolumnar junction to recede toward the os, with the glandular epithelium being replaced by squamous epithelium. The area traveled by this slowly moving junction is called the transformation zone and is the site where cervical cancer occurs. The squamous metaplasia is guided by unique cells expressing p63 and keratin 6 that lie between the squamous cells and glandular cells. These cells trigger the emergence beneath themselves of squamous reserve cells, which then proliferate and differentiate (212). HPV DNA has been found in prostatic tissue and semen, although conflicting reports exist on the prevalence and biological significance of this association. Metastases of HPV-induced lesions also contain HPV DNA. However, whereas some BPVs can infect lymphocytes and establish metastatic tumors, similar evidence for HPV remains inconclusive (213).

The duration of HPV infections and diseases is highly variable, ranging from weeks to years. Lesion regression is not random. The regression of treated lesions may be accompanied by the regression of the untreated lesions, a phenomenon consistent with a host response (214, 215).

HPV can be recovered not only from skin and mucosal lesions but also from the normal surrounding tissues, where lesions usually relapse (191, 192). Remarkably, HPV-6 or -11 DNA is found in a third to a half of the hair follicles plucked from the pubic and perianal area of patients with condylomata acuminata (194). The vast majority of HPV infections are latent (185, 194, 216, 217). Many of the cervical HPV infections are transient, especially in younger women, and usually contain a low copy number of HPV DNA (82, 129, 218). Transient expression is more common during the luteal phase than during the follicular phase of the menstrual cycle. In the cervix, HPV persistence was estimated to be 9.8 months, with high-risk HPV persisting longer than low-risk HPVs, HPV-16 persisting the longest (12.4 months) (218).

Latency and Cancer

Infection with a high-risk HPV is associated with a risk of developing CIN that appears to be specific for each

genotype, but the effect of viral load on persistence and disease progression is more complex than previously appreciated, and the relevance of multiple concomitant HPV infections is largely unknown (195, 219). The issue of persistence itself is fraught with methodological difficulties, including the frequency of sampling and the detection sensitivity of the HPV DNA assay used (195). Therefore, it is unclear if immune responses are capable of fully clearing an HPV infection, or if the virus persists in a latent state, undetected and producing no viral particles (66, 195). The viral and cellular factors that induce, maintain, or abrogate latency are unclear, other than immunosuppression, which facilitates the reactivation of latent infection (129, 195, 217). Anecdotally, the initiation of an oral contraceptive treatment has been associated with a flare-up of genital warts.

The oncogenic properties of several animal papillomaviruses, such as BPV and cottontail rabbit papillomavirus, were experimentally demonstrated long ago (52). A cancer precursor can be produced by infecting human foreskin with HPV-16 prior to grafting in SCID mice (59).The anogenital HPV types most capable of immortalizing and transforming cells *in vitro* are those associated with cervical cancer. Different viral genes, notably E6 and E7 from high-risk types, have transforming, immortalization, and oncogenic potential, especially in combination, when introduced into cell lines or transgenic animals. These genes interfere with cell cycle regulation and apoptosis (see "Virology" above) (Table 2).

HPV-induced malignant transformation seems to result from a complex series of events that are independent of viral particle production. Integration of HPV DNA into the host genome appears to be associated with the progression from high-grade CIN to cancer. Integration occurs in the majority of invasive cervical carcinomas but is rare in benign and premalignant lesions; however, this may be an underestimation (28, 195, 220). Integrated and episomal forms may coexist in the same cells. The sites of viral integration in the host genome are not random and exist on a variety of chromosomes. They contribute to oncogenicity by being in proximity to cellular oncogenes or by creating areas of host genomic instability in the manner they integrate (220). The site of integration in the viral genome is typically restricted between the 3′ end of E1 and the 5′ end of E2, resulting in disruption, deletion, or inactivation of the E2 ORF. These changes appear to disrupt the viral and cellular regulatory controls of viral gene expression mediated by the E2 proteins. This allows the free transactivation of the E6/E7 promoter by several cellular transcription factors (28, 29, 35, 195, 219, 221). Integration may also disrupt the E1, E4, E5, L1, and L2 ORFs, but the E6 and E7 ORFs are typically spared. Viral integration does not appear to be necessary for progression to malignancy, given that up to a quarter of invasive cervical cancers contain only viral episomes (195). In addition, on the rare occasions when low-risk oncogenic HPVs such as HPV-6 and -11 have been associated with squamous cell cancers, the viral genome undergoes deletions, mutations, and amplifications in the URR but remains episomal. DNA sequence heterogeneity exists within each genotype, and molecular variants have different oncogenic potentials (67).

Although the E6 and E7 genes are necessary for immortalization and malignant transformation, they are not sufficient. Hybrid clones from the fusion of cell lines containing HPV-16 do not necessarily retain their malignant phenotype, as would be expected if this phenotype was solely dependent on the presence of the viral genes (222). Therefore, intracellular molecular mechanisms, so far mostly unidentified, are often capable of keeping the oncogenic process in check. However, one internal signal that has been identified is the CDKN2A gene, which codes for the p16INK4A protein. Its role is to inhibit the cell cycle by inactivating cyclin D1-CDK4 or cyclin D1-CDK6 complexes, thus down-regulating cyclin E. Methylation, mutation, or deletion of CDKN2A is necessary for E6 to exert its immortalizing effects. E7 by itself increases p16INK4A levels, which makes this protein a potentially useful biomarker of malignancy, but it also circumvents the inhibiting effect p16INK4A would have by stimulating the downstream cyclins A and E (Fig. 6). Additional genes participating to S-phase progression are upregulated by high-risk HPV infection; they include MCM, PCNA, Ki67, and they have been used as potential biomarkers of malignancy progression. Another of these activated genes is paradoxically p14ARF, which by inactivating MDM increases the levels of p53, but E6 is available to accelerate through ubiquitination the degradation of p53, the net effect being a decrease in p53 (28). External signals also contribute to the downregulation of the transcription and oncogenesis of the high-risk HPVs. These cytokines include tumor necrosis factor alpha (TNF-α), alpha interferon (IFN-α), IFN-β, IFN-γ, retinoic acid, and tumor growth factor β (TGF-β) (28, 29, 219, 221, 222). Some potential mechanisms have been identified. The transcription complex AP-1 is a heterodimer made of c-Jun and c-Fos (Fig. 5) or c-Jun and Fra I. Only c-Jun and c-Fos can bind and participate in the activation of the HPV E6/E7 promoter. Tumor necrosis factor alpha and retinoic acid induce the Fra I gene, thus favoring the formation of the c-Jun–Fra I complex, the net effect being downregulation of the HPV promoter.

Other endogenous factors associated with malignant transformation have been identified. Cellular oncogenes (e.g., c-myc and c-ras) may be activated by the nearby integration of viral DNA into the host chromosome. This is significant because the E7 gene can cooperate with activated ras to induce transformation in primary rodent cells.

Methylation has emerged as an important epigenetic mechanism of carcinogenesis control by causing the inactivation of viral and cellular genes and their regulatory sequences (36, 195, 223). The promoter region of several tumor suppressor genes can be inactivated by aberrant methylation of the GpC islands. E7 is thought to be able to stimulate the transcription of some DNA methylases, causing methylation of the pRB pocket proteins and inhibition of E2F-regulated transcription.

Various cytogenetic abnormalities have been observed in cervical carcinomas, such as alterations of chromosome 1 and allelic losses on the short arm of chromosome 3 or 17 or the long arm of chromosome 11 (219, 222). This loss of heterozygosity may eventually lead to the disappearance of suppressor genes; for example, p53 is located on 17p. E6 and E7 genes can induce chromosomal abnormalities *in vitro*. Chromosome markers have been used to study the cellular clonality of cervical lesions. Low-grade lesions are typically polyclonal, while high-grade lesions seem to be oligoclonal, or monoclonal if concomitant with an invasive cancer. Invasive cancers are monoclonal.

Genetic host factors appear important in the development of cervical cancer. For example, the family-attributable risk of cervical cancer is higher in full sisters than in adopted or half-sisters of women with cervical cancer (224). One case control study found that HLA allele combinations af-

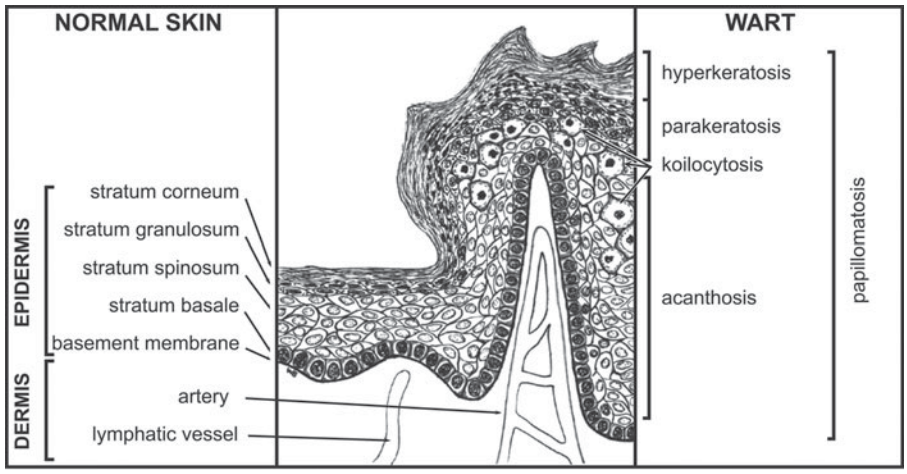

FIGURE 8 Drawing of the histologic features of normal skin and of a wart.

fect cervical SCC risk, with effects all less than 2-fold in magnitude (128). The HLA class I alleles A*0301, B*4402, and Cw*0501 conferred an increased risk, and B*1501 conferred a decreased risk. The combination of HLA class II alleles DRB1*1101 and DQB1*0301 decreased the risk, while the combination of DRB1*1302 and DQB1*02 increased the risk. In high-order interaction effects, most of the combinations containing B*4402 or DQB1*0301 were associated with an increased risk. Of interest, the combination B*4402-DRB1*1101-DQB1*0301 increased the risk of cancer 10-fold. All these associations were also observed in cervical adenocarcinoma and vulvar squamous cell cancer.

Numerous exogenous factors play a putative role in HPV-related malignant transformation, including UV and X-ray irradiations, smoking tobacco, steroid hormones, chlamydia, and viruses like HIV, herpes simplex virus, and Epstein-Barr virus. However, much remains to be learned about their contributions and mechanisms of action.

Histopathology

Epidermal basal cells are arranged around a central, putative basal stem cell in groups of approximately 10. These groups define the base of the epidermal proliferative unit. One may speculate that HPV needs to infect the basal stem cell to become established. As the putative basal cell divides, the viral genome replicates. Some viral copies are transmitted to the daughter cell that is eventually thrust upward to form a parabasal cell. As they ascend toward the surface of the epithelium, the keratinocytes stop dividing but continue to differentiate (Fig. 8). Differentiation is a process associated with the expression of different keratins. Viral replication is independent of cellular replication and may continue as the keratinocytes reach the upper layers of the stratum spinosum. However, to replicate, the virus has to activate and appropriate the cell molecular replicative machinery. The viral particles are shed with the desquamating cells of the stratum corneum.

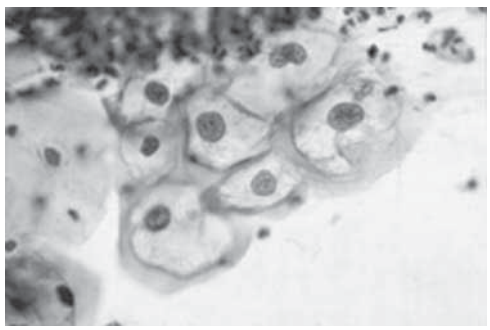

FIGURE 9 Pap smear demonstrating koilocytic atypia. In contrast to the histologic specimen, by cytology koilocytes have one or two relatively large, smooth, oval nuclei, surrounded by a very large halo of amorphous substance. (Courtesy of Clara E. Mesonero, Cape Cod Hospital, Cape Cod, MA.)

The typical histologic features of benign cutaneous HPV disease include thickening of the stratum spinosum (acanthosis), stratum granulosum (parakeratosis), and stratum corneum (hyperkeratosis) (Fig. 8). The persistence of nuclei in the stratum corneum is also a feature of parakeratosis. The presence of koilocytes (koilocytosis) (from the Greek *koilos*, cave) is strongly indicative of HPV infection. The koilocyte is an enlarged keratinocyte that develops in the upper layers of the stratum spinosum and in the stratum granulosum. It is recognized by a clear halo that surrounds a central nucleus, shriveled and shrunken on tissue sections and enlarged on cytologic smears (Fig. 9). The cell may be binucleated or the nucleus may be bilobar. The cytoarchitecture of the lesion is papillomatous, with fronds of epidermis extending toward both the dermis and the surface. A combination of the same histologic characteristics can be found in the mucosal epithelia infected by HPV. Because such epithelia are not keratinized, hyperkeratosis is not a frequent or prominent feature. In an inverted papilloma, the papillomatosis is frank but strictly limited to the dermis, thus giving a flat lesion. HPV type also contributes to variations in the histology of lesions. Figures 10 and 11 illustrate the histology of different types of warts.

HPV infection of the cervical squamous epithelium can result in a range of histopathological entities for which different classification schemes have been designed (Fig. 12). This evolution reflects the many fine points of the surgical pathology diagnosis and the debates that surround them. What follows is only a crude description of the squamous epithelial changes.

Cervical flat condylomas are benign lesions characterized by the presence of koilocytes. The abnormal proliferation of the basal layer is one of the features that define

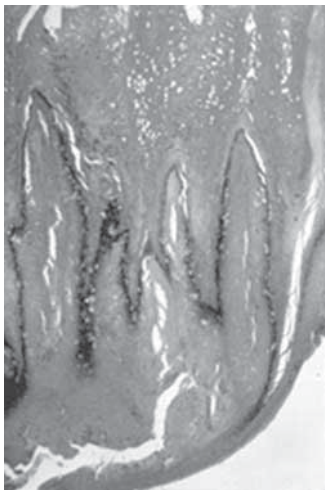

FIGURE 10 Histology of a cutaneous wart (hemalum-eosin stain; low-power view). The darkly stained layer is the stratum granulosum. Koilocytes are profuse and disrupt the stratum granulosum. The persistence of nuclei in the stratum corneum is a feature of parakeratosis. Note the thick stratum corneum. (Courtesy of Clara E. Mesonero, Cape Cod Hospital, Cape Cod, MA.)

intraepithelial neoplasia. These lesions, which are preinvasive cancers, are graded according to the extent of basal-type cell proliferation. Proliferation up to the lower third of the epithelium is mild dysplasia or CIN 1, up to the upper two-thirds is moderate dysplasia or CIN 2, and up to the full epithelium is severe dysplasia/carcinoma *in situ* (CIS), or CIN 3. In CIS, the full epithelium is abnormal. The local breach of the basal membrane by the epithelial cells characterizes an invasive SCC. The other features of this abnormal cellular proliferation are more prominent as the severity of the lesion increases. They include an enlarged cell nucleus/cytoplasm ratio, coarse granularity of the nucleus, numerous and abnormal mitotic figures, and some abnormal

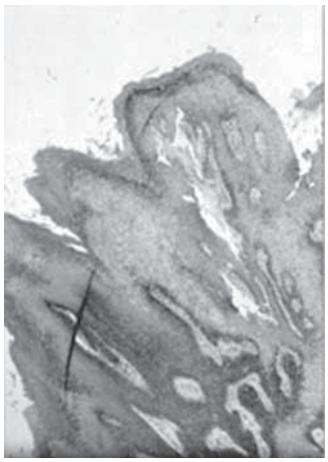

FIGURE 11 Histology of condyloma acuminatum (hemalum-eosin stain; high-power view). The figure demonstrates many koilocytes in the stratum spinosum. Koilocytes are relatively large cells with a shrunken, irregular nucleus surrounded by a halo. (Courtesy of Clara E. Mesonero, Cape Cod Hospital, Cape Cod, MA.)

and necrosed cells (atypia defines these abnormal or atypical cellular changes). Koilocytosis may be absent, particularly in the higher grades, which otherwise display anisocytosis. The cytological equivalents of cervical condyloma and CIN are LSIL for cervical condyloma and CIN 1 and HSIL for CIN 2 or 3 (Fig. 12). As a parallel, some pathologists now use the terminology of low-grade CIN (L-CIN) and high-grade CIN (H-CIN) for histology. In 2012, the LAST project of the College of American Pathologists and the American Society for Colposcopy and Cervical Pathology proposed to apply the terminology LSIL and HSIL to both cytology and histology of the cervix, as well as to use p16INK4A immunostaining to better classify formerly CIN2 lesions (225).

From a normal-appearing cervix to CIS there is a graded risk of progression toward invasive cancer (226). This gradient can be superimposed on a gradient of increased prevalence of high-risk HPV types (Fig. 12) (64, 227, 228). Although it would seem that lesions slowly progress through each stage of CIN before becoming invasive, a rapid progression can occur without recognition of the intermediate stage, and progression from a normal cervix to H-CIN can occur in as little as 2 years (129).

Several histologic events accompany the spontaneous regression of warts (214, 215, 229). Spongiosis of the basal cells occurs and a lymphomonocytic infiltrate develops in the dermis and lower epidermis. Necrosis and apoptosis are present in the epidermis; the dermis exhibits focal thrombosis and hemorrhages. The lymphocytic infiltrate is primarily T cells, with CD4 cells predominating. The lymphocytes display the isomorphs CD45RA+ and CD45RO+, indicating the presence of naïve and memory cells, respectively. Lymphocytes, Langerhans cells, and keratinocytes are immunologically activated, displaying HLA-DR and ICAM-1 molecules. In addition, the lymphocytes exhibit high-affinity interleukin 2 (IL-2) receptors (CD25). These histologic changes are consistent with a delayed-type hypersensitivity reaction. An increase in natural killer (NK) cell activity has also been associated with resolution of CIN 1. Thus, the cellular immune system appears to be important in HPV disease regression.

Immune Responses

The immunology of HPV infections is imperfectly understood in its details (215, 230-234). The frequency, severity, and persistence of HPV infections and diseases in immunocompromised patients have long indicated that immunity, cell-mediated immunity in particular, contributes to the development and resolution of HPV infections (113, 139, 229, 230, 235). For example, primary immunodeficiencies such as ataxia-telangiectasia, Wiskott-Aldrich syndrome (due to the WAS protein deficiency), and common variable immunodeficiency are risk factors for the development of extensive verrucosis (113). The original model for an association between a genetic alteration and verrucosis has been epidermodysplasia verruciformis, a genodermatosis associated with inactivating mutations in two genes, EVER1/TMC6 and EVER2/TMC8, that code for endosomal zinc transporter proteins that are part of the NF-κB cell signaling cascade essential to the immune response (189, 190, 236, 237). However, in the past decade a great number of other conditions have been added to the list. They include the WHIM syndrome (warts, hypogammaglobulinemia, infections, myelokathexis) related to a CXCR4 deficiency, the WILD syndrome (warts, immunodeficiency, lymphedema, anogenital dysplasias), XHIGM1 (X-linked hyper-IgM syndrome type 1), hyper-IgE syndrome, atopic dermatitis,

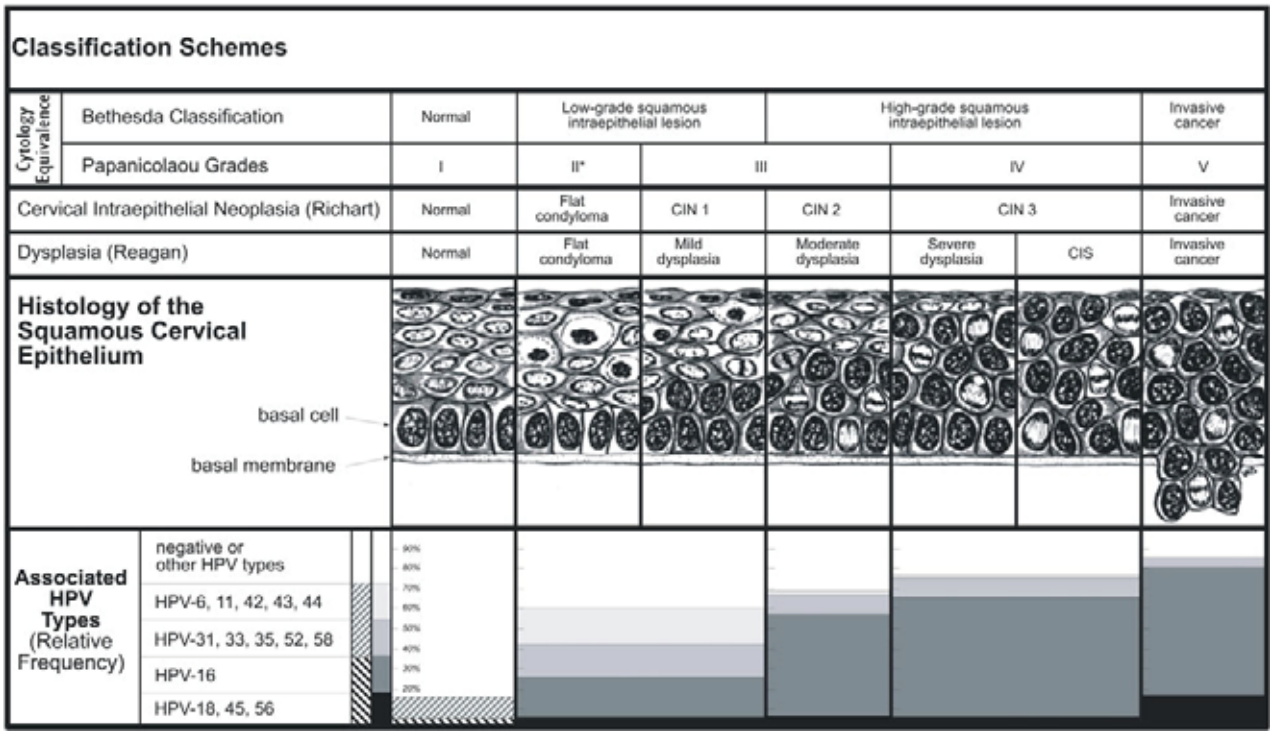

FIGURE 12 Diagrammatic representation of the nomenclature, histologic features, and distribution of associated HPV types in HPV-related cervical lesions. The dysplasia and the CIN classifications are primarily histologic classifications that are also used for cytology, whereas the Bethesda classification is designed mainly for cytology (see text for details).
*This category in the Pap classification also included atypical squamous cells of the Bethesda classification.

Netherton syndrome, severe combined immunodeficiency (SCID) due to defective γc chain of the IL-2 receptor, adenosine deaminase (ADA), or the Janus kinase 3 (JAK-3), deficiencies in the dedicator of cytokinesis 8 (DOCK8) protein (the cause of autosomal recessive hyper-IgE syndrome) or of GATA2 (a transcription factor), and others (113, 230).

The keratinocyte is the first barrier to an HPV infection because it expresses various Toll-like receptors (TLR) able to recognize pathogen-associated molecular patterns (PAMPs) (232). One of them, TLR-9, recognizes double-stranded CpG-rich DNA and in turn stimulates the production of several cytokines, TNF-α, IL-8, CCL2, CCL20, CXCL9, monocyte chemotactic protein 1 (MCP-1), vascular endothelial cell growth factor, IL-5 and -8, retinoic acid, TGF-β, and type 1 interferons (IFN) (IFN-α, -β), as well as IFN-γ-inducible protein 10 (IP-10 [CXCL10]) and IFN -γ. Some of these responses are consistent with the notable mononuclear cell infiltrate present in the dermis and epidermis during regression of a wart or condyloma. These cells allow the development of a local and systemic specific immune response, a predominantly Th1 cytotoxic response. However, HPV has ways to blunt this part of the immune response. HPV-16 E6 and E7 may abrogate the production of TLR-9. HPV also dysregulates the interferon response in different ways (215, 232, 233). E6 likely interferes with the TYK2 kinase, preventing its binding to the interferon receptor and blocking the phosphorylation of TYK2, STAT1, and STAT2 an essential step for the activation of the JAK-STAT cascade. HPV E7 can bind to the p48/IRF9 complex, preventing its translocation to the nucleus where it would bind the IFN-stimulated gene factor 3 (ISGF3) to form the transcription complex for the IFN-specific response element (ISRE). HPV E7 also interacts with IRF1 and not only blocks the expression of IFN-β but also downregulates the transporter associated with antigen processing 1 (TAP1), IFN- β, and monocyte chemoattractant protein 1 (MCP1).

The respective roles in antigen presentation of the Langerhans cells, which are the epidermal professional antigen-presenting dendritic cells, and the keratinocytes, which upon immunologic activation can also behave as antigen-presenting cells, are unclear. However, the density of Langerhans cells is generally decreased in nonregressing warts, which may be related to the downregulation of E-cadherin by HPV E6 and E7. HPV capsids do not activate Langerhans cells, and dermal dendritic cells and macrophages may have a greater role in presenting the HPV antigens to the lymphocytes. Nevertheless, HPV can reduce antigen presentation by downregulating this process, inhibiting the expression of proteasome subunits of the major histocompatibility complex (MHC) class 1, TAP, and also by reducing the cytoplasmic trafficking of MHC-1 molecules and stopping them in the Golgi apparatus (215, 232, 233).

Cervical cancer progression can be associated with a shift induced by E7 in the cytokine profile from a Th1 to a Th2 response. This is mediated through the production of IL-10, which represses the production of HLA-1, stimulates the production of HLA-G, and inhibits natural killer cells (233). The presence of infiltrating FOXP3+ Treg, likely with specificity to E6 and E7, is associated with a poor survival because they blunt the antitumor response (234).

Apoptosis, or programmed cell death, allows a cell to abort a viral infection and end tumor cell proliferation, but HPV can evade this antiviral and antitumor response. HPV E5 affects both apoptosis-activated caspase pathways by inhibiting TNF-related apoptosis inducing ligand (TRAIL) and CD95L (233). It also downregulates the CD95 receptor and the formation of the death-inducing signaling complex (DISC). HPV E6 participates in this inhibition by stimulating the degradation by ubiquitination of pro-apoptotic proteins FADD, procaspase-8, or c-Myc.

The humoral response to the early viral proteins is typically modest or absent in most patients. Patients with invasive cervical cancer are the most significant exception; approximately half of whom develop antibodies to HPV-16 E6 and E7 peptides or fusion proteins. Otherwise, the most consistent and strongest immune response in HPV-infected patients is to the native conformation of the L1 protein present in virions or recombinant VLPs (18, 238). These L1 antibodies can be neutralizing, but this requires them to be of high titer, which is rarely the case, as the HPV VLP vaccine studies have shown (239–242). The immune response to native L1 is typically type specific (10).

Lymphoproliferative responses to different HPV proteins can be detected in some patients with HPV infections, especially those with cervical preinvasive cancers. The most consistent and strongest of these responses are against E6 and E7 (18, 19). The same proteins are also the targets of a cytotoxic T-cell response (18, 19). NK cell cytotoxicity against HPV-infected keratinocytes is present in epidermodysplasia verruciformis and CIN (105, 106). The precise nature and role of these responses in disease persistence and resolution largely remain to be established and explained in the context of the immunogenetic factors that govern HPV disease (189, 237).

CLINICAL MANIFESTATIONS

Cutaneous Warts

Three major types of cutaneous warts are recognized: deep plantar warts, common warts, and flat or plane warts (196). Deep plantar warts (verrucae plantaris), also termed myrmecias (from Greek, ant hill), are usually solitary lesions preferentially located on the weight-bearing surfaces of the foot. These deeply set painful lesions can be 2 mm to 1 cm in diameter. They interrupt the ordered pattern of surrounding rete ridges and look like a circular, disorganized bundle of keratotic fibers, sometimes containing dark speckles, surrounded by a slightly raised keratotic ring. The black dots represent thrombosed capillaries (Color Plate 34). Occasionally, the lesion is completely covered by the keratotic ring and can take on the appearance of a corn or callus. Tenderness and the appearance of punctate, thrombosed capillaries after paring the horny layer with a scalpel are features distinguishing a deep plantar wart from a callus or a fibrokeratoma. Occasionally, myrmecias can develop on the palms of the hands.

Common warts (verrucae vulgaris) are usually multiple, well-circumscribed, exophytic, hyperkeratotic, round papules with a coarse surface ranging in size from 1 mm to 1 cm (Color Plate 35). They are normally found on the dorsum of the hand, between the fingers, and around the nail bed. They can also grow under the nail bed, causing nail loss (onycholysis). Mosaic warts, a variant of common warts, may be found on several areas of the foot, including the sole, the knuckles, and around the toenails (Color Plate 36). They appear as multiple, confluent, shallow, slightly raised, keratotic lesions. These painless lesions coalesce and may cover several square centimeters. Filiform warts are common warts usually located on the face, particularly the lips, nares, or eyelids. They are thin, fleshy projections, usually 1 to 3 mm in diameter and a few millimeters long. Cutaneous horns are an uncommon presentation of common warts and may be confused with keratoacanthoma, basal carcinomas, SCC, actinic keratoses, or seborrheic keratoses. The warts of meat handlers usually resemble typical common warts (243). Common warts may be confused with nevi, seborrheic keratoses, acrochordons (skin tags), molluscum contagiosum, keratoacanthomas, lichen planus, lichen nitidus, syringomas, dermofibromas, or SCC. In the healthy host, malignant transformation of common warts into Bowen's disease, verrucous carcinoma, or SCC is a rare event and may be related to the presence of high-risk oncogenic HPV types in the lesions (244).

Most cutaneous warts are asymptomatic. Bleeding and pain, particularly with deep plantar warts, may occur in pressure areas. The natural history of cutaneous warts is poorly defined. In children, the rates of spontaneous resolution after 1 and 5 years of follow-up are 50% and 90%, respectively (206, 207). Individual deep plantar and common warts take about 1 month to disappear spontaneously. They blacken, become inflamed, or involute. In any given patient, two-thirds of the warts that resolve spontaneously will do so within the first 2 months.

Plane or flat warts (verrucae planae) present as multiple, flat, small, asymmetric, smooth papules with a pink to tan color. They are found mostly on the face (especially the chin and eyelids), neck, and hands of children. Taller lesions are sometimes called intermediate warts. Lichen planus and freckles may be confused with flat warts. Reddening, swelling, and itching of lesions announce simultaneous flat wart regression, a process that lasts 2 to 7 weeks.

In immunocompromised patients, HPV-associated lesions may take on appearances other than that of common warts, such as warty keratoses (Color Plate 37), or epidermodysplasia verruciformis-like plaques distributed in the sun-exposed areas (dorsum of the hands, face, scalp, and neck). In these patients, malignant or benign lesions such as SCC, basal cell carcinomas, Bowen's disease, keratoacanthoma, or actinic keratoses can contain HPV (185).

Epidermodysplasia Verruciformis

Epidermodysplasia verruciformis is an autosomal recessive genodermatosis causing an abnormal susceptibility to Betapapillomaviruses (189, 236, 237). It results from the mutation of two adjacent genes, EVER1/TMC6 and EVER2/TMC8 on chromosome 17 that code for two endosomal zinc transport proteins, which participate in NF-κB cell signaling (190). Of note, lesions that resemble epidermodysplasia verruciformis are have been observed in HIV-infected patients, solid organ allograft recipients, and patients with tuberculoid leprosy (189, 245). The disease is characterized by the appearance of flat wart-like lesions, red to brown plaques (Color Plate 38), or pityriasis versicolor-like lesions over the face, the torso, and the extensor surfaces of the extremities, usually during the first decade (189, 236, 237, 246). Over 20 different HPV types have been isolated from epidermodysplasia verruciformis lesions (Table 1), often coexisting in the same patient. HPV-3 is uniquely associated with lesions resembling large flat warts. The prevalence of plantar and common warts appears to be increased in these patients. In about half the patients, beginning before the age

of 30 years and extending over the following decades, lesions in sun-exposed areas, primarily those associated with HPV-5, -8, or -47, undergo premalignant and malignant changes. They form papillomas, seborrheic keratoses, and SCC. The malignancies remain locally invasive and slow growing, unless they have been exposed to local irradiation. Biopsy is useful in the diagnosis and management of epidermodysplasia verruciformis.

Anogenital HPV Diseases

The full evaluation of a patient with potential anogenital HPV disease should include a history that reviews symptoms, particularly those pertaining to emotional well-being, sexual intercourse, urination, and defecation. Duration of symptoms and treatment received should be asked along with age at first intercourse, number of past and current sexual partners, sexual practices, and use of barrier methods of contraception. The patient's history of other sexually transmitted diseases (STD) (including HIV infection) and treatments should be recorded, as should the histories of anogenital warts, intraepithelial neoplasias, and cancers in the sexual partners. The physical examination should be done aided, if necessary, by the application for 3 to 5 minutes of 3% to 5% acetic acid (white vinegar) and the use of a colposcope (a biomicroscope offering several magnifications, ranging typically from ×6 to ×40, and a long focal length) or a powerful magnifying glass. The female internal genitalia should be examined with a speculum. A Pap smear may be obtained at that time prior to further interventions (247). A diagram displaying the anatomic location of the lesions will facilitate evaluation and monitoring. A digital rectal examination completes the examination in immunocompromised patients (HIV patients and transplant recipients) as men who have sex with men, or if anal symptoms are present. Immunocompromised women should have a pelvic examination.

External Genitalia and Anus

Condylomata acuminata (singular, condyloma acuminatum; from the Greek *kondyloma*, knuckle, knob, and Latin *acumen*, sharp point), also known as anogenital warts or venereal warts, are slightly hyperkeratotic, firm, exophytic papules that are flesh colored to dark gray and are either sessile or attached by a broad, short peduncle (Color Plate 39). On the skin, small lesions tend to be smooth, round papules with an accentuation of the skin ridges. Larger skin lesions or mucosal lesions are more cornified and may have an irregular, jagged, mulberry-like, or pointed surface contour. Sizes usually vary from a millimeter to 2 cm but can reach several square centimeters, particularly when several lesions coalesce like cobblestones to form a plaque. Individual lesions are devoid of hair.

In men, the predominant location of lesions is the penile shaft in circumcised individuals and the preputial cavity otherwise (114, 248). One percent to 25% of patients will have urethral warts that are usually located between the meatus and the fossa navicularis (114). Warts rarely extend beyond the distal first 3 cm, and involvement of the proximal urethra and bladder is exceptional. Meatal eversion or the use of an otoscope or pediatric nasal speculum facilitates the inspection of meatal warts. Although perianal warts can occur in heterosexual men, they are much more common in homosexual men (249–251). The scrotum, perineum, groin, and pubic area are rarely affected.

In women, the great majority of lesions are found over the posterior introitus, including the fourchette, spreading toward the labia minora and majora and clitoris. In decreasing order of frequency, the perineum, vagina, anus, cervix, and urethra represent less than 25% of the usually affected sites (114, 248). Young girls with anogenital warts should have a careful examination of the anus and genitals, and according to the findings or age of the patient, proper referrals need to be made (87, 90, 92).

For both sexes, anoscopic examination is recommended if there is a history of receptive anal intercourse, if perianal warts are present, or if the patient has anal symptoms. Because lesions rarely extend beyond the pectinate line, sigmoidoscopy is not ordinarily performed. An oral examination is indicated for the presence of associated oral warts.

Itching, burning, and even pain and tenderness are the most common symptoms of condylomata acuminata (248). Yet approximately three-quarters of patients are asymptomatic (248). Nonetheless, the disease has a significant psychosexual impact on about half of the patients before or after treatment. During pregnancy or immunosuppression, warts may increase in number or size and may obstruct the birth canal. The natural history of condyloma acuminatum is poorly defined, but 10% to 20% of patients will experience a spontaneous remission within 3 to 4 months of presentation. Genital warts disappear spontaneously by involution.

Diagnosis and Differential Diagnosis

The poxvirus infection, molluscum contagiosum is the disease most frequently mistaken for condyloma acuminatum. Lesions of molluscum contagiosum are usually small, circular, well-defined, dome-shaped, sessile papules (see Chapter 19). They are flesh to wax colored, not pigmented, with a smooth or dotted surface containing a central depression from which a cheesy material can be extruded. Their anatomic distribution helps the differential diagnosis because they predominate over the pubis and can extend to the trunk. Condyloma lata of syphilis are relatively large, smooth, sessile, moist, flat-topped lesions that are few in number. The medical history and serology help with the diagnosis. Nodular scabies appears as red, scaly or crusted, deeply set nodules accompanied by an intense pruritus, particularly at night. The differential diagnosis includes other dermatologic conditions. Hidradenoma papilliferum, encountered on the keratinized vulva, resembles a large wart. Acrochordons (skin tags) are soft, skin-colored tumors. Epidermoid cysts and angiokeratomas can be found on the scrotum. Lichen planus, lichen sclerosus et atrophicus, lichen nitidus, or syringomas manifest as small, planar lesions that may be difficult to recognize. Small penile warts in the corona glandis may be very difficult to distinguish from a normal anatomic variant called hirsutoid papillomatosis (pearly coronal papules, papillae corona glandis). Whether the sometimes painful papillae that may be present on the vulvar introitus represent the female equivalent of hirsutoid papillomatosis or are lesions caused by HPV (i.e., vulvar papillomatosis) has been controversial. In both sexes, one may encounter sessile papules or macules that have a brown to slate blue pigmentation (Color Plate 40). These lesions are particularly important to recognize because they may represent benign condylomas infected with HPV-6 or -11 (252), nevi, seborrheic keratoses, or intraepithelial neoplasias infected with HPV-16 or -18 (252). The poor correlation between appearance and histology or HPV type argues for the biopsy of these lesions. Biopsy should also be considered if lesions are large, bleed, appear in plaques, or are in unusual locations. There should be a lower threshold to biopsy immunosuppressed or immunodeficient patients.

Subclinical HPV lesions, either revealed by the colposcope or the application of acetic acid, are more often than

macroscopic lesions associated with high-risk HPV infections (253). Subclinical lesions appear mostly as small acetowhite papules. In male partners of patients with condyloma acuminatum, one-third to more than two-thirds have detectable lesions that are seen only with the aid of the colposcope, and a smaller percentage is seen by acetowhitening alone (253). On the vulva, HPV infection may produce white patches that are enhanced or exposed after application of acetic acid. In some surveys, over 80% of women with or exposed to condyloma acuminatum were found to have subclinical infections. The clinical significance of acetowhite lesions is unknown, in part because acetowhitening lacks specificity and possibly sensitivity for diagnosis of HPV infection on the external genitalia (254). Acetowhite lesions may also be caused by menstruation or recent coitus. Subclinical lesions are usually transient, and diagnosis and treatment of asymptomatic subclinical diseases are not recommended (247, 253). Furthermore, dermatitis, folliculitis, candidiasis, psoriasis, lichen sclerosus et atrophicus, lichen simplex chronicus, and trauma can produce the same symptoms of pain, discomfort, and itching in the absence of visible lesions. The distinct entity of vulvar vestibulitis, also called focal vulvitis, does not appear to be related to HPV infection.

Vagina and Cervix

Vaginal warts manifest as either spiked condylomas, white, keratinized nodosities centered on a capillary loop, or flat condylomas (114). The latter may be confused with the occasional normal anatomic variant of micropapillary projections that are located over the distal third of the vagina and extend over the introitus (vulvar papillomatosis).

Cervical warts are found in less than 10% of women with genital warts (114). Three variants are recognized: the flat condyloma (Color Plate 41) and the rarer exophytic and inverted (endophytic) condylomas. These lesions are also acetowhite, and colposcopic examination facilitates their identification (Color Plate 42). Because colposcopy is not entirely reliable, several biopsies of suspicious-looking areas are usually required for evaluation. Nevertheless, in the developing world, simple naked-eye visual inspection aided by acetic acid is able to contribute effectively to the prevention of cervical cancer. This screening can be further improved when followed by HPV DNA testing.

Preinvasive and Invasive Malignant Lesions

The clinical appearance of HPV-related preinvasive and invasive malignant cervical lesions is defined by colposcopic criteria that attempt to match histopathology (Color Plates 41 and 42) (255). Most of the lesions of CIN and SCC develop in the transitional zone. Usually not accessible to visual inspection, the glandular epithelium is vulnerable to HPV infections, especially by HPV-18, which may cause adenocarcinoma (65).

Depending on histologic grade, CIN lesions may regress, persist, or progress at various rates. According to a meta-analysis, the risks of progression over 2 years to HSIL from ASCUS and LSIL are 7% and 21%, respectively (226). Over 2 years, the risks of progression to invasive cancer are low, 0.25% for ASCUS, 0.15% for LSIL, and 1.4% for HSIL. In contrast, the rates of regression to normal cytology or histology are high, 68% for ASCUS, 47% for LSIL, and 35% for HSIL, and independent of duration of follow-up.

Intraepithelial neoplasias at the anogenital sites are often referred to by the acronyms of PIN (penile intraepithelial neoplasia), VIN (vulvar intraepithelial neoplasia), VAIN (vaginal intraepithelial neoplasia), and AIN (anal intraepithelial neoplasia). Intraepithelial neoplasias can coexist in different anatomic locations, thus reflecting the multicentric nature of the disease. Clinically, these lesions may present on the skin as pigmented papules, leukoplakia, or red macules (252). Bowenoid papulosis is a distinct clinicopathological entity made of the multicentric aggregation of pigmented papules, ranging from dark red to dark blue, with the histologic features of an intraepithelial neoplasia and the cytoarchitecture of a condyloma (Color Plate 40). It may evolve into Bowen's disease, which is a carcinoma *in situ* that presents as a red to brown, flat, scaly plaque with an irregular surface but well-demarcated borders. On the glans penis, Bowen's disease is known as erythroplasia of Queyrat. High-risk HPVs are typically associated with these premalignant conditions. Natural history data on intraepithelial neoplasias of the external genitalia are inadequate. Like CIN, VAIN and early vaginal cancer are best recognized with the colposcope, after the application of acetic acid; biopsy is necessary to establish a diagnosis.

On the external genitalia, warts may exceptionally evolve into verrucous carcinoma, a slow-growing, large, locally invasive SCC (244). Some authors distinguish Buschke-Loewenstein tumor (also called condylomatous carcinoma or giant condyloma) from what others consider to be a less aggressive form of verrucous carcinoma (Color Plate 43) (244). Buschke-Loewenstein tumors look like large condylomas with a locally invasive behavior.

Recurrent Respiratory Papillomatosis

An altered cry in infants or hoarseness of voice in older individuals is the presenting clinical manifestation of recurrent respiratory papillomatosis (102, 103). Stridor or respiratory distress may also be present. On laryngoscopic examination the lesions are fungating and smooth (Color Plate 44), and are encountered specifically where the ciliated and squamous epithelia are juxtaposed. The lesions are also found along the tract of tracheostomies. The severity of the disease is inversely related to the age of onset; tracheostomy is required more often in children (14%) than adults (6%) and extralaryngeal spread is also more common in children (31%) than adults (16%). Lesion growth and extension toward the lung compromise the respiratory tract, and frequent surgical treatment may be needed to prevent asphyxiation. About one in five patients requires more than 40 lifetime operations. Most lesions contain HPV-6 or -11. Lesions containing HPV-11 might be more aggressive. Malignant transformation resulting in verrucous carcinomas or SCC occurs in less than 10% of individuals, but the risk is the greatest if the lesions were previously treated with irradiation. Disease extension to the lung or the aerodigestive tract carries a risk of malignant transformation as high as 85%. Recurrent respiratory papillomatosis appears to be surprisingly rare in the HIV-infected population.

Miscellaneous

The different HPV-related lesions of the oral cavity are clinically similar but can be differentiated by histology (62, 256-258). The most common are oral squamous cell papillomas (or squamous papillomas) and the closely related entity oral condylomata acuminata, both caused by the same HPV types, mostly HPV-6, -11, and -16. Verrucae vulgaris lesions are less common and are caused by cutaneous HPVs, such as HPV-2, -4, and -57. HPV DNA, particularly HPV-16 DNA, can be found in various oral intraepithelial neoplasias, verrucous carcinomas, and SCC, as well as in prolifer-

TABLE 4 Diagnostic methods for anogenital HPV infection[a]

HPV diagnostic method (name of FDA-approved diagnostic kit)	Tested material or subject	HPV type determination	Availability to the clinician	Relevance for patient management	Sensitivity	Specificity
Clinical examination (cervix not included)						
With naked eye	Patient	No	+++++	+++++	+	++
And with acetic acid application	Patient	No	+++++	++	++	+
And examination with colposcope	Patient	No	+++	+++	++	++
Colposcopy	Patient	No	+++	+++++	+++	++
Cytology						
Cervical	Swabbing, washing	No	+++++	+++++	++	++
Noncervical	Swabbing	No	++[b]	?+++[c]	++	++
Histology						
Light microscopy	Tissue	No	+++++	+++	++	+++
Electron microscopy	Tissue	No	+	+	+	++++
Immunocytochemistry	Swabbing, washing, tissue	No	++	++	++	++++
Nucleic acid detection						
In situ hybridization	Tissue	Possible	+	++	++	++++
FDA-approved assays (Hybrid Capture II HR DNA; Cervista HPV HR; Cervista HPV 16/18; COBAS 4800 HPV; APTIMA HPV)	Swabbing, washing, tissue	Possible[d]	+++++	+++	++++	++++
Non-FDA-approved PCR and RT-PCR, RFLP, microarrays, LAMP, NASBA, TMA assays	Swabbing, washing, tissue	Possible	+	?	++++/+++++	+++/+++++
Serology	Serum	Yes	+	+	++	+++

[a] + to +++++, from the lowest to the highest magnitude.
[b] This rating takes into account the fact that the technique needs to be associated with high-resolution anoscopy.
[c] In HIV patients and in men who have sex with men.
[d] Possible for HPV-16 and -18 with some of the FDA-approved tests. Non-FDA approved kits do exist for the genotyping of genital HPVs.

ative verrucous leukoplakia (62, 258). Focal epithelial hyperplasia (Heck's disease) manifests as multiple, asymptomatic, 1- to 5-mm, soft, sessile papules distributed preferentially on the lower lip but also on the buccal mucosa, lower lip, tongue, and gums (62, 257, 258). The lesions usually disappear over time. In HIV-infected patients oral hairy leukoplakia is caused by Epstein-Barr virus infection, although coinfection with HPV does occur (62). HPV infection may cause papillomas and malignant tumors of the conjunctiva, a risk increased by a concomitant HIV infection (183, 184).

LABORATORY DIAGNOSIS

The role of the laboratory includes both confirming the clinical diagnosis by histology or screening precursor and malignant lesions in the cervix by cytology and detecting HPV DNA in cervical samples to aid in the screening and prevention of cervical cancer (Table 4). A practical method for HPV isolation by *in vitro* culture is not yet available.

Cytology and Colposcopy

Cytology has been applied to the diagnosis of HPV infections of the penis, vulva, and anus, but cervical cytology, in the form of the Papanicolaou (Pap) smear, remains the most extensively used. Cells can be collected by washings, but swabbing or scraping is standard. The optimal method of collection combines the use of the Ayre spatula to scrape the exocervix and the Cytobrush® to sample the endocervix (259). The Cervex-Brush® ("broom"), which is designed to sample both areas simultaneously, does not perform as well in traditional cytology (259). The sample can be placed directly on a glass slide and fixed (traditional cytology). An alternative and now widely used approach is to place the sample (ThinPrep with the PreservCyt™ Solution; Hologic) or the head of the broom (SurePath™; Becton, Dickinson and Company) in a liquid fixative (260, 261). The cells are dispersed and are either gathered on a filter and transferred to the glass slide (ThinPrep) or centrifuged on a gradient, with the suspended cells allowed to sediment on the glass slide (SurePath). Both liquid-based preparation technologies appear to offer approximately equivalent performance characteristics that are at least equal to those of traditional cytology (260-265). Their higher cost and longer processing time are offset by the ability to test the cervical sample for HPV DNA, as well as the possibility to combine the tests with a computer-automated imaging system that analyzes the slide and selects the areas of interest for the cytologist to review.

Koilocytes and dyskeratocytes (small, very keratinized squamous cells with orange cytoplasm and nucleus atypia) are hallmarks of cervical HPV infection (Fig. 9). However, atypical squamous metaplasia and, to a lesser degree, dyskeratosis correlate independently with the presence of HPV

infection by PCR better than koilocytosis (266). Cytology is at least 10 times less sensitive than PCR for the diagnosis of HPV infection and should not be used for such a purpose (see "Epidemiology" above).

Over the years different classification schemes have been applied to the Pap smear (Fig. 12) and multiple ones remain in use globally. Their shortcomings led to a consensus effort in the United States that resulted in the Bethesda system in 1988, which was revised in 2006 (www.asccp.org/ConsensusGuidelines/tabid/7436/Default.aspx) (267). The Bethesda system has three main components: a judgment on the adequacy of the specimen, a categorization of the smear with a codified descriptive diagnosis, and a set of guidelines for management and follow-up. Squamous cell abnormalities fall into four categories: (i) atypical squamous cells (a) of undetermined significance (ASC-US) or (b) for which HSIL cannot be excluded (ASC-H), (ii) LSIL, (iii) HSIL, and (iv) SCC. The performance characteristics of the Pap smear are difficult to determine accurately. The estimated sensitivity ranges from 30% to 87% and the specificity ranges from 86% to 100% (268). It is clearly an imperfect tool for the diagnosis of preinvasive and invasive cervical cancer, but it is still a preferred screening test because it is easy to obtain, has low cost, and with periodic repetitions diminishes the chance of missing invasive cancer. The liquid-based system has allowed the use of computer-aided diagnosis systems to attempt to increase speed, accuracy, and reproducibility while keeping the cost down (269, 270). Automated screening can be implemented for initial screening (triage), thus diminishing technologist workload considerably (269, 270). The BD FocalPoint™ Slide Profiler (Becton, Dickinson and Company) imaging system is FDA-approved for primary screening of conventional slides (approved in 1998) and liquid-based SurePath prepared slides (2003), while the ThinPrep Imager (Hologic) is FDA-approved (2003) for the primary screening of liquid-based ThinPrep prepared slides (274–276). The BD FocalPoint Guided Screening Imaging system the FDA approved in 2008 is a further development of the BD FocalPoint Slide Profiler that eliminates the need for a cytopathologist to review a sampling the slides identified as negative by the machine (270–272).

Abnormal cervical cytology is usually supplemented by colposcopy to visually inspect the lesion and select a biopsy site for histologic analysis (255). After applying 5% acetic acid, the cervix is examined with a colposcope. The application of an iodine solution (Lugol solution) may be included in the examination (Schiller test). Various scoring schemes have been developed to try to differentiate between typical condylomas, low-grade and high-grade intraepithelial neoplasia, and cancer. They rely to various degrees on color, shape of lesion margins or surface contours, appearance of the vessels (punctation and mosaicism), and iodine staining (Color Plates 41 and 42) (255). Like all visually based diagnostic methods, colposcopy suffers from limited accuracy compared to histology, as well as poor intra- and interobserver agreement, particularly when contiguous diagnostic categories are concerned (273, 274). However, it is an indispensable tool for identifying the biopsy sites. Taking additional biopsies when multiple lesions are present clearly improves the confirmation rate by histology of a cytologic diagnosis of HSIL (275).

The colposcopic technique has been applied to the screening of patients at risk of anal cancer. The scoring system developed for the cervix is as effective for the anus in allowing discrimination between L-CIN and H-CIN (150, 276–279).

Cervicography generates a picture of the cervix for review by an expert (255), but like colposcopy, it suffers from poor intra- and interobserver agreement and has not performed well (274, 280, 281). It may retain a place in cancer screening in areas with low resources, to augment the performance of visual inspection with acetic acid (VIA) (282). Given its feasibility and affordability, VIA has been widely implemented in low- and middle-income countries in Asia and Africa, in which WHO currently recommends a screening strategy of HPV testing, followed if necessary by VIA and immediate treatment with cryotherapy or loop electrosurgical excision procedure (LEEP) if cryotherapy is inappropriate (283, 284).

Histology

Histology is the "gold standard" for confirming diagnosis of HPV disease and is the most important among the laboratory resources available to the clinician. However, sampling error, sample size, and fixation artifacts can all contribute to inconclusive results. Histology is not an appropriate tool for the diagnosis of HPV infection because it selects only small areas for biopsy, and most HPV infections are asymptomatic associated with a normal histology (73–76, 192).

Histologic criteria enabling the diagnosis of the different HPV diseases (Figs. 10 and 11) are neither absolute nor easy to define. Interobserver variability is a substantial limitation that affects histology and cytology equally. Even among well-trained pathologists, interobserver agreement on cervical biopsies is only moderate, and it is worst for CIN 2 (129, 285). These difficulties have motivated the use of the LSIL and HSIL nomenclature for the histologic diagnosis and the addition of the p16INK4A immunostaining to better classify CIN2 lesions (225).The clinical identity of a lesion cannot always be established from the histology. For example, whereas anogenital warts, flat warts, and myrmecias are easily differentiated, other cutaneous warts are more difficult to identify (286-288). Histologic criteria have been elaborated for HPV-diseases of the head and neck, vulva, and anus (289, 290).

Transmission electron microscopy is of little use in the diagnosis of HPV lesions, but it may reveal the presence of intranuclear viral particles that are typically organized in crystalline arrays or pseudoarrays (5, 291–295). HPV intranuclear particles have been noted in histologically or colposcopically normal cervical samples (295).

Immunocytochemistry

Denaturation of papillomavirus virions by boiling and mercaptoethanol treatment exposes an antigen that is shared among papillomaviruses. Antibodies to the common papillomavirus or genus-specific antigen are commercially available and have been used for the immunocytochemical diagnosis of HPV infection (Fig. 13). The sensitivity of this test is limited and varies with lesion type (20, 296).

Immunocytochemistry plays an important role in cervical cancer screening to complement histopathology by identifying the presence of cellular and viral proteins that indicate a greater oncogenic risk. The detection of the E6, E7, E4, and L1 viral proteins, for example, has been used to indicate the onset of cancer progression (297, 298). Among the cellular proteins, p16INK4A, the minichromosome maintenance protein 2 (MCM2), topoisomerase II alpha (TOP-O2A), Ki-67 (a marker of cell proliferation), MYBL2 (a member of the MYB proto-oncogene family that encodes DNA-binding proteins) have been targeted to improve the identification of the risk of progression (298–300). The

biomarkers Ki-67, the pair MCM2 and TOPO2A (ProExC™ assay; Becton-Dickinson), and p16INK4A have slightly different performance profiles for the diagnosis of ASC-US, LSIL, and HSIL (298, 301). The identification by immunocytochemistry of biomarkers that improve the histologic diagnosis has led to the LAST project recommendations, which include not only the use of the LSIL/HSIL nomenclature for histology but also the use of the p16INK4A stain in instances when a CIN2 diagnosis is considered (225). A p16INK4A positive lesion would be classified as HSIL, while a p16INK4A negative lesion would be degraded as LSIL. The immunocytochemistry of biomarkers also applies to HPV-associated lesions at other anatomic sites (302–304).

Nucleic Acid Detection Assays

In situ hybridization (ISH) enables the visualization of HPV nucleic acids in cells or tissues under the microscope, typically a paraffin-embedded tissue section. Probes can be sense or antisense. Antisense probes offer the advantage of binding to both viral DNA and mRNAs, and either of these targets can be selected by denaturing and pretreating with RNase or not (305, 306). The advantages of *in situ* hybridization include the ability to type the infecting HPV, to detect double infections, to distinguish integrated (speckled nuclear signal) from episomal (diffuse nuclear signal) infections, and to relate infection to associated histopathological features. Different commercial tests have been developed, but none are approved by the FDA (306).

PCR diagnosis offers the highest analytical sensitivity and specificity for the detection of HPV DNA (307, 308) and also permits typing. A variety of methods have been described for mucosal and cutaneous HPV types that are based on the use of either general (also called consensus or generic) primers or a mixture of primers (degenerate primer system) (298, 307–313). Typing can be done by retesting the sample with type-specific primers or by applying type-specific probes to the amplicon. Other technologies developed for viral nucleic acid detection and genotyping (298) include real-time PCR, reverse line blot hybridization assays, restriction fragment length polymorphisms (RFLP), microarrays, loop-mediated isothermal amplification method (LAMP), nucleic acid sequence based amplification (NASBA), and transcription mediated amplification (TMA).

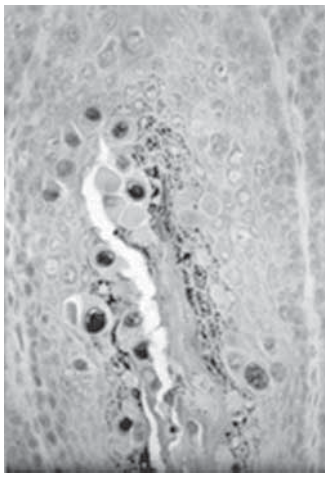

FIGURE 13 Immunocytochemistry of condyloma acuminatum with an antibody directed against the common papillomavirus antigen (high-power view). Several intense nuclear signals are visible. (Courtesy of Clara E. Mesonero, Cape Cod Hospital, Cape Cod, MA.)

MicroRNAs are noncoding cellular RNAs that contribute to cell regulation, and at least two of them, miR-34a and miR-125b, can be altered in an HPV infection and used as biomarkers (314). The expression of miR-34a is directly controlled by p53 and behaves as a tumor suppressor while miR-125b intervenes in the immune response and apoptosis. Similarly, the upregulation the TERC gene that codes for a component of the telomerase, an enzyme activated in rapidly dividing cells, could be useful as a cellular biomarker of lesion progression (304).

The FDA-approved assays are all designed to detect high-risk mucosal HPV (Alphapapillomavirus genus) nucleic acids (DNA or RNA) from cervical cells to achieve at least one of three goals: a) primary screening of cervical cancer; b) reflex testing of specimens diagnosed as ASC-US to identify the women who need to be referred to colposcopy; and c) cotesting with cytology for women 30 years and older to guide their management. Five tests are currently available and are part of automated platforms (see Table 5) (298, 315).

Direct comparative studies of the performance of these assays for the detection of CIN2+ show equivalent sensitivities in the 91% to 96% range but specificities lower than 29% and in decreasing order for the APTIMA, COBAS, HC2, and Cervista assays (316, 317).

All these assays are approved for two basic indications: the reflex-testing of ASC-US samples and adjunct HPV DNA testing in cervical cytology samples of women 30 years and older. In addition, the COBAS HPV assay has been FDA-approved (2014) for the primary screening of women 25 years and older.

Serologic Assays

There are no serologic assays satisfactory for the clinical diagnosis of HPV infection. The most consistently sensitive assays are VLP-based enzyme-linked immunosorbent assays used chiefly for the diagnosis of HPV-6, -11, -16, and -18 infections (18, 318, 319). They cannot identify much more than half to two-thirds of infected subjects if they are to retain a high specificity and detect an immunoglobulin G (immunoglobulin A to a lesser extent) immune response that decreases only a little over time (242, 320–322). Hence, they measure HPV exposure. This makes them useful, if imperfect, seroepidemiological tools to measure past and present infection (18, 319). These assays and their derivatives, such as the Luminex® bead technology, are investigational, but they have been also useful for the assessment of the immune response following immunization (319, 322–324). Assays vary in their ability to detect various neutralizing antibodies or binding antibodies (319, 324, 325).

PREVENTION

General

No specific environmental precautions have been developed for the control of HPV infections, and there is limited knowledge about the resistance of HPV virions to physical and chemical agents (60, 61). However, surgical instruments in contact with areas potentially infected by HPV should be properly sterilized according to standard procedures developed for viruses. Disposable materials should not be reused. Contaminated surfaces can be disinfected with household

Table 5 Diagnostic tests for the detection anogenital HPV nucleic acids

Test name (Manufacturer)	Sample	Target, Assay principle	Nature of probes, HPV types detected	Signal detection, Result interpretation	Comments
Hybrid Capture II High Risk DNA assay (Qiagen)	Cells[a,b] or extracted DNA	HPV DNA DNA:RNA hybridization	RNA 16, 18, 31, 33, 35, 39, 45, 51, 52, 58, 59, 68	Antibody to DNA:RNA complexes, conjugated to alkaline phosphatase Positive/negative/indeterminate	• Does not differentiate HPV types • Retesting is necessary for the indeterminate results • Cross-react with some of the non-oncogenic HPV types (false-positives) • Does not assess for the presence of human cells in the sample • Scalable to automation with Rapid Capture System (Qiagen)
Cervista® HPV HR (Hologic)	Extracted DNA using GenFind® (Hologic) [b]	HPV DNA E6/E7 Invader chemistry and hybridization	Three oligonucleotides: 2 invader HPV E6/E7 specific for each genotype and 1 universal hairpin FRET[c] signal 16, 18, 31, 33, 35, 39, 45, 51, 52, 58, 59, 66, 68	Upon binding, a flapping 5' end of the type specific probe is released by a Cleavase enzyme. This flapping end will act as an invader probe to the universal hairpin FRET signal oligonucleotide. This again triggers the Cleavase enzyme to separate on the probe the fluorophore from its quencher. The process repeats itself, generating a large signal. Positive/negative	• Does not differentiate HPV types • May cross-react with HPV types 67 and 70 (false-positives) • Contains control probes against histones to ascertain the presence of cells • Scalable to automation with the Cervista® High Throughput Automation (HTA) system
Cervista® HPV 16/18 (Hologic)	Same as above	Same as above	Same as above but limited to types 16 and 18	Same as above	• Differentiates between HPV type 16 or 18 • May cross-react with high copy number of HPV type 31 (false-positive) • Same as above otherwise
COBAS 4800 HPV (Roche)	Cells [b]	HPV DNA L1 Real-time PCR	8 DNA primer pairs HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 58, 59, 66, 68	Three HPV DNA probes are cleaved during the extension phase of the PCR, releasing a distinct fluorophore from its quencher Quantitative results with cut-offs: HPV-16 positive/negative; HPV-18 positive/negative; other HR[d] HPV positive/negative	• Contains primers/probe against •-globin to ascertain the presence of cells • The amplicon incorporates dUTP instead of dTTP allowing the degradation of any contaminant amplicons with uracyl-N-glycosidase (which will not destroy the thymidine-containing target DNA) • Automated platform
APTIMA HPV (Hologic)	Cells [b]	E6/E7 mRNA HPV transcripts Transcription-mediated amplification whereby MMLV reverse transcriptase generates cDNA and T7 RNA polymerase generates copies	Type specific single-stranded RNA chemiluminescent probes + internal control 16, 18, 31, 33, 35, 39, 45, 51, 52, 58, 59, 66, 68	Hybridization protection assay in which the hybrid RNA:cDNA are protected and the chemiluminescent label can be detected. Positive, negative, invalid	• Automated platforms Tigris DTS and Panther

bleach (5.25% sodium hypochlorite) diluted 1:10 in water. Cryoprobes have been responsible for transmission of warts from patient to patient, thus making sprays preferable for cryotherapy.

Electrosurgery and laser surgery are accompanied by the release of smoke that contains HPV DNA that may lead to the contamination of the surgeon's mucosal membranes or of the operating room surfaces (326, 327). Whether this leads to higher prevalence of hand and nasopharyngeal warts is unclear (328, 329). Smoke evacuation systems and the wearing of gloves, gown, mask, and goggles are recommended when performing these procedures (326, 327, 329–331).

Covering the warts with dressings while at the swimming pool has been useful for the prevention of plantar warts (108). However, there is conflicting data regarding the use of protective footwear in communal areas of swimming pools, public showers, or gyms (107, 109, 110).

Strong evidence indicates that male condoms are effective in reducing the transmission of genital HPV (127) (see "Epidemiology" section). For example, in a prospective study of 82 college-age, initially virgin women, 100% consistent male condom use in partners was associated with a 3-fold decreased risk of cervical, vulvar, and vaginal HPV infection, compared to 5% or less use (332). Among condom users, genital warts are more likely to occur in the anatomical area not covered by the condom (197). Furthermore, in a randomized study of 125 couples of women with CIN, there was a greater rate of CIN (1.5-fold) and HPV DNA clearance (5.7-fold) in the women from the couples assigned to condom use (333) as well as higher (1.9-fold) rate of clearance of HPV-associated flat lesions in the males, and the clearance effect of condoms in males was seen only in the couples with concordant HPV types (334, 335). In a prospective study of 82 college-age, initially virgin women, 100% consistent condom use, as opposed to 5% or less use, was associated with a statistically significant (3-fold) decreased risk of cervical, vulvar, and vaginal HPV infection (332). Condom use can be recommended after treatment of one sexual partner for HPV disease.

Patients with epidermodysplasia verruciformis and immunodeficient or immunosuppressed patients with cutaneous lesions should avoid UV light exposure to minimize the risk of malignant conversion.

There are no validated isolation procedures of HPV-infected individuals. There is no evidence that school activities should be restricted for children with recurrent respiratory papillomatosis.

Passive Immunoprophylaxis

Although the potential efficacy of passive immunoprophylaxis using hyperimmune globulins raised against VLPs has been demonstrated with experimental animal papillomavirus infections, no data are available for HPV infections (336).

Chemoprophylaxis

It is possible to prevent the relapse of anogenital warts with either podofilox or 5-fluorouracil (5-FU) (see "Treatment" below), but effective chemoprophylaxis against new HPV infection is not available. Carrageenan, a readily available polysaccharide already included in some vaginal lubricants, has shown excellent microbicidal activity *in vitro* and in an animal model (337, 338). Clinical studies are ongoing to assess its prophylactic effectiveness. In contrast, the spermicide nonoxynol 9 has been shown to potentiate HPV transmission (337).

Active Immunization

HPV vaccination, introduced in 2006, is the most important advance in HPV care and is expected to sharply reduce the occurrence of anogenital infections and cancers. The current vaccines are based on expressing the gene coding for the major capsid protein (L1), which spontaneously assembles to form a virus-like particle (VLP) that has the same appearance and antigenic properties as the native virions, but is not infectious. Papillomavirus VLPs induce a strong humoral response that neutralizes the papillomavirus of the homologous type (238, 318). This response is much higher than the one triggered by a natural infection, which possibly confers only a weak protection (242, 339). Studies done in an animal natural host validated the concept, which was then applied to human vaccination (336, 340–342). In a pivotal randomized controlled trial, a monovalent HPV-16 VLP vaccine was shown to completely prevent persistent cervical HPV-16 infection compared to adjuvant at 1.5 years of follow-up (343).

There are currently three vaccines on the market (344-347). The first to appear in 2006 was a quadrivalent vaccine (Gardasil/Silgard; Merck & Co., Inc.) directed at HPV types 6, 11, 16, and 18 (345). In all likelihood, it will be replaced completely by a nonavalent extension (Gardasil®9; Merck & Co., Inc.), FDA-approved in 2014, that adds types 31, 33, 45, 52, and 58 (these types are related to HPV-16, except 45, which is related to HPV-18) (347). A bivalent vaccine, covering only types 16 and 18 (Cervarix; GlaxoSmithKline) was FDA-approved in 2009 (344). HPV-6 and -11 cause about 85% to 95% of genital warts (348), while HPV-16 and -18 account for about 70% of cervical cancers and over 90% of the other cancers associated with HPV (including vulva, vagina, penis, anus, oropharynx) (97, 100, 349–351). The five types added to Gardasil-9 represent 30% of the genotypes associated with CIN2/3 and 25% of those causing CIN1, while HPV-16 and -18 are present in 50% and 30% of these lesions, respectively (352). Gardasil and Gardasil-9 are made in yeast (*Saccharomyces cerevisiae*) while Cervarix is made in a baculovirus expression system. The other major difference is the adjuvant, amorphous aluminum hydroxyphosphate sulfate for Gardasil and Gardasil-9 (225 and 500 μg, respectively), and aluminum hydroxide (500 μg) plus 3-O-desacetyl-4'-monophosphoryl lipid A (MPLA) (50 μg) for Cervarix. All vaccines are delivered in a 0.5 ml per dose intramuscular injection (deltoid muscle), in a three-injection series given at time 0, 1 month (Cervarix) or 2 months (both Gardasil), and 6 months.

Efficacy in Clinical Trials

The efficacy of the current vaccines was established in registrational trials conducted in adolescent and young women aged 16 to 26 years. Gardasil when compared to the placebo (adjuvant alone) was found to have a 99% efficacy (95% CI, 96–100) against genital warts, a 98% efficacy (95% CI, 93–100) in preventing CIN2/3 or adenocarcinoma *in situ* (AIS), and an efficacy of 100% (95% CI, 83-100) against VIN2/3 and VAIN2/3 caused by HPV-6, -11, -16, or -18 (353). The efficacy of Cervarix compared to placebo (a hepatitis A vaccine containing the same adjuvant) was 93% (95% CI, 80–98) for the prevention of CIN2/3 or AIS caused by HPV-16 or -18 (354).The evaluation of Gardasil-9 generated the same levels of neutralizing antibodies against HPV-6, -11, -16, and -18 as Gardasil and had an efficacy of 97% (95% CI, 81–100) in preventing CIN2+, AIS, VIN2+, or VAIN2+ caused by HPV types 31, 33, 45, 52, or 58 (347).

Full vaccine efficacy is provided only against the infections or diseases caused by the HPV genotypes contained in the vaccine with which the subjects have not been infected before (355). Consequently, HPV vaccination should be initiated before the onset of sexual activity to have its greatest impact. Because conducting efficacy studies in children would have required an impractical long period of follow-up for a clinical efficacy endpoint, HPV neutralizing antibody levels were used as correlates of efficacy in "bridging" studies of Gardasil and Cervarix-9. In studies conducted in girls and boys aged 9 years through 15 years, antibody titers were demonstrated to be higher than in the female adolescents and young adults enrolled in other studies (356–363). The same successful bridging studies were conducted with Gardasil-9 (362).

Gardasil was also effective in 16- to 26-year-old males in preventing genital warts, 90% (95% CI, 69-98), and any grades of AIN in MSM, 78% (95% CI, 40–93), caused by the HPV vaccine-types (364). There are no data yet on vaccine efficacy in the HIV-infected population, but these subjects are capable of seroconverting in response to Gardasil or Cervarix, although specific antibody levels that may be 2- to 5-fold lower than in HIV-seronegative individuals (365-368). The significance of this finding is unclear because antibody thresholds below which protection is lost have not been established. When directly compared in HIV-seropositive subjects, Cervarix was more immunogenic than Gardasil in women, but equivalent in men (369, 370).

Gardasil has been evaluated in older women (24–45 years) and the serologic response was lower with age, an observation also made with Cervarix in 15- to 55-year-old women (371, 372). Vaccine efficacy was demonstrated with Gardasil but only by combining external genital warts with CIN as the endpoint (371). This has led to approval of the vaccine in women up to 45 years old in Canada but not in the United States.

At the end of 2015, none of the available vaccines had shown failure of clinical efficacy in the follow-up of the phase 2/3 studies, including 8.5 years of follow-up with the original HPV-16 VLP vaccine (373, 374). Gardasil has retained full protection against genital warts and CIN1-3 at 5 years (373, 374). Specific neutralizing antibody levels have been sustained for up to 7 years, with a slight decline after 8 years, but no occurrence of disease, in boys and girls immunized between ages of 9 and 15 years (375). Cervarix was also still effective at 6.4 years, and at 9.4 years the limited data were still encouraging (376, 377). When compared directly, Cervarix induced higher levels of antibodies to HPV-16 or -18 than Gardasil, likely on the account of the stronger adjuvant (378, 379). The clinical significance of these differences is unknown at present. The same can be said of the stronger cross-reactive antibody response induced by Cervarix against nonvaccine types 31, 33, and 45 (380). These differences, if present, did not appear to be as pronounced when the two vaccines were compared in the same study (381). Furthermore, in Australia where HPV immunization was mostly conducted with Gardasil, some cross-protection was noted against nonvaccine types 31, 33, and 45 HPV prevalent cervical infection, with a vaccine efficacy of 58% (95% CI, 26–76) (382).

Efficacy in the General Population

There is now sufficient experience with HPV vaccination throughout the world to measure its substantial clinical impact through various observational studies. In Australia, where Gardasil was used extensively to immunize girls, the cervical prevalence of HPV strains dropped from 28.7 to 6.5% between the prevaccination (2005–2007) and post-vaccination (2010–2012) periods (382). This was also likely accompanied by a herd-immunity effect. In the United States, a large survey of over 4,000 females aged 14 to 59 years showed a drop by 44% in the prevalence of vaginal swab HPV DNA for the vaccine types (6, 11, 16, and 18) from the period 2001 to 2006 to the period 2007 to 2010 in the cohort of 14- to 19-year-old girls, the ones most likely to have received the vaccine but not in older women (75). More recently, the same survey was revisited, focusing on 14- to 34-year-old females and comparing the 2001–2006 pre-vaccine period to the 2009–2012 postvaccine eras (383). Vaccine HPV DNA prevalence declined significantly by 64% in the 14- to 19-year-olds and by 34% in the 20- to 14-year-olds. A 68% reduction in prevalent HPV-16 and -18 cervical infections was found in countries where the vaccine coverage rate was more than 50% (85). These benefits have also been observed in the HPV infection rates of other anatomic areas, such as the anus and oral cavity (384).

Epidemiologic surveys have almost all demonstrated drastic reductions in the prevalence of anogenital warts. A recent meta-analysis reported a 61% reduction between the pre- and postimmunization periods in 13- to 19-year-old girls for those countries where the vaccination coverage was superior to 50% (85). The magnitude of the effect increased over time and was accompanied by evidence of herd-immunity only for those countries with more than 50% vaccine coverage. Similarly, HPV vaccination has led in reductions in the prevalence of CIN or SIL in the United States (385), Australia (386, 387), Denmark (388), and Scotland (389). Although the HPV vaccines appear to be devoid of any therapeutic activity, an unexpected benefit of HPV vaccination has been an apparent reduction in the recurrence rates of HPV cervical diseases and possibly AIN (390, 391).

Safety

The HPV vaccines are safe and generally well tolerated, with the adjuvants contributing to most of the early adverse reactions (344, 345, 392, 393). The most common side effects are local injection-site reactions of mild to moderate intensity, lasting about 3 days on average, although some vaccinees experience systemic symptoms within 1 week after immunization. In a direct comparison trial of Gardasil and Cervarix, the respective rates were 72% and 92% for any local pain, 26% and 44% for any local erythema, and 22% and 36% for swelling at the local injection site, 40% and 50% for fatigue, 42% and 46% for headaches, 27% and 33% for gastrointestinal symptoms, 16% and 22% for arthralgia, and 11% and 14% for temperature of 37.5°C or more (394). Discontinuation from participation in the clinical trials for adverse reactions was noted in only 0.2% of the almost 12,000 patients receiving either Gardasil or placebo (adjuvant alone) (393). In a similar analysis including almost 30,000 patients receiving either Cervarix or various controls, the withdrawal rates were 0.17% and 0.09%, respectively (392). Adverse reactions are more frequent after the first injection than with the subsequent ones (344, 345). Owing to its double amount of adjuvant compared to Gardasil, Gardasil-9 has slightly more frequent local (but not systemic) adverse reactions, 90% versus 84% for pain, 34% versus 26% for erythema, and 40% versus 29% for swelling (347).

An extensive and worldwide series of postlicensure monitoring programs have been put in place (392, 395-399).

The Vaccine Adverse Event Reporting System (VAERS), a passive surveillance system that allows the reporting by laymen and health professionals of any presumptive problems with vaccines, promptly identified an excess of fainting episodes at the time of the immunization (400). This led to the recommendation of keeping the vaccine recipient under observation for 15 minutes after the vaccine injection. Twenty-six cases of anaphylaxis, none lethal, were also reported, 17 occurring after the first dose. Known allergic reactions to components of the vaccine, in particular baker's yeast for Gardasil and latex for Cervarix (contained in the cap of prefilled syringes) are contraindications for the vaccine administration.

The HPV vaccination is contraindicated in pregnant patients; any vaccination series interrupted by pregnancy should be completed after delivery. Nevertheless, pregnancies occurred during the clinical trials, and among about 1,700 women exposed to either Gardasil or Cervarix during their pregnancy, no excess of abnormal pregnancies, spontaneous abortions, or major birth defects were found (401, 402). These reassuring observations were replicated elsewhere (403, 404). Lactation is not a contraindication for vaccination.

In the postmarketing surveillance programs, particular attention has been directed at detecting any possible risk of thromboembolic or autoimmune diseases, in particular Guillain-Barré syndrome and demyelinating diseases (397, 405–410). None has been found except, in the largest survey done to date, among French females aged 13 through 16 years, including 842,000 who had received the HPV vaccine (Gardasil for 93 %) and 1,414,596 who had not; the only strong risk identified among 14 different conditions was for Guillain-Barré syndrome, with a hazard ratio of 4.0 (95% CI, 1.8–8.7) (411). This represented a low incidence of 1 to 2 cases per 100,000 vaccinees, with mostly no severe sequelae.

Recommendations

The FDA has approved Cervarix for the prevention of CIN1-3, cervical cancer, and AIS caused by HPV-16 and -18 in females aged 9 through 25 years. The indications for Gardasil are broader. For subjects 9 to 26 years of age, they include the prevention in the appropriate sex of (a) cancers of the cervix, vulva, vagina, and anus caused by HPV-16 and -18; (b) cervical AIS, CIN1-3, VIN1-3, VAIN1-3, and AIN1-3, caused by HPV-6, -11, -16, and -18; and (c) external genital warts caused by HPV-6 and -11. For Gardasil-9, the indications are similar to those of Gardasil, but to HPV-16 and -18, they add types 31, 33, 45, 52, and 58.

The Advisory Committee on Immunization Practices (ACIP) issued recommendations for HPV vaccination (247, 346, 412). Any of the three existing vaccines can be used in females, who should receive the vaccine between ages 11 and 12 years, although it can be given as early as 9 years. A catch-up immunization is recommended for 13- to 26-year-olds not vaccinated previously or who did not complete the three-dose series. For males only Gardasil and Gardasil-9 are recommended. Vaccination should be started at ages 11 to 12 years but can be given as young as 9 years. A catch-up immunization is recommended for 13- to 21-year-olds not vaccinated previously or who did not complete the three-dose series. Vaccination of the 22- through 26-year-old males should be considered.

The three vaccines are to be administered according to a three-dose schedule, at time 0, at least 1 to 2 months after the first dose, and at least 6 months after the first dose. Inadequate doses, or doses administered too soon, should be readministered. If the series is interrupted, it simply needs to be completed, not restarted. The HPV vaccines can be co-administered with the other age-appropriate vaccines (tetanus, diphtheria, acellular pertussis, and quadrivalent meningococcal conjugate vaccines) but using separate syringes and separate injection sites. It is expected that one subject immunized with one of the HPV vaccines should be able to mount a protective immune response and not incur additional adverse reactions if subsequently immunized with a different HPV vaccine. This was confirmed in females aged 12 to 26 years old who received Gardasil-9 after receiving Gardasil (363).

In addition to the general population, special populations are particularly targeted for HPV vaccination. They include MSM for whom vaccination is recommended up to age 26 years old if they have not been previously vaccinated or have not completed the three-dose series. Both females and males immunocompromised due to transplantation, medication, or HIV infection should be immunized up to the age of 26 years old. Those with a history of sexual abuse or assault should follow the general recommendation, but the immunization should be started as early as 9 years of age.

Immunization is also recommended for those patients with an abnormal Pap test, a known HPV infection, anogenital warts, or HPV-associated lesions. The HPV vaccines have no therapeutic effect, but they still will protect against the HPV types that have not yet infected the patient. No prior HPV DNA or antibody testing is either available or recommended. Most importantly, HPV vaccination does not modify the recommendations for continued cervical cancer screening (see below).

Prevention of Perinatal and Congenital Infection

Delivery by cesarean section in pregnant women with condyloma acuminatum is not indicated for the prevention of transmission to the baby (102, 103). There is no consensus on the use of cesarean section for the delivery of babies whose mothers have had a child with recurrent respiratory papillomatosis.

Prevention of Anogenital Malignancies

Several guidelines issued in 2012 are available for the screening of cervical cancer (Table 6) (413). The two notable innovations are the initiation of screening at age 21 years and the role of high-risk HPV DNA testing, either as an adjunct to cytology or alone as the primary screening method. Guidance is also available on how to approach abnormal screening tests both online (http://www.asccp.org/Guidelines-2/Management-Guidelines-2) and in print (267, 414). In addition, with the recent introduction (2004) of high-risk HPV DNA as the primary screen, interim guidelines have been issued on how to approach the results (415, 416). The cervical cancer screening and management of women infected with HIV should be differentiated (http://aidsinfo.nih.gov/guidelines/html/4/adult-and-adolescent-oi-prevention-and-treatment-guidelines/0) (417). It is important to emphasize that at present HPV vaccination status does not change any of these guidelines.

Screening strategies for anal cancer based on anal cytology have been proposed (418, 419). The procedure, which can be self-administered, consists of the introduction into the anal canal, more than 2 cm from the anus, of a Dacron swab moistened with saline or water or of a cervical Cytobrush. The collected material is processed like a conventional or liquid-based Pap smear. Nevertheless, anal cytology

TABLE 6 Summary of cervical cancer screening guidelines[a]

Parameter	Organization	Guideline
When to begin Pap test screening?	ACS, ACOG, USPSTF	At age 21 years
How often?	By cytology	
	ACS, ACOG, USPSTF	Every 3 years for women ages 21–65 years
	By HPV co-testing (cytology + HPV test)	
	ACS, ACOG, USPSTF	Not to be used below the age of 30 years
		Every 5 years for women aged 30-65 years
	By Primary hrHPV testing	
	USPSTF, ACS	Not recommended
	ACOG	Every 3 years, starting at age 25 years
When to stop?	ACS, ACOG, USPSTF	After age 65 years with adequate negative prior screening
	ACS, ACOG	AND no history of CIN2+ within the last 20 years
	USPSTF	AND not otherwise at high risk for cervical cancer
When to screen after age 65 years?	ACS, ACOG	If history of CIN2/3 or AIS, continue screening for 20 more years
	USPSTF	If never screened, does not meet criteria for adequate prior screening, or history of adequate prior screening cannot be accurately documented, screening should continue for at least 20 years after spontaneous regression or appropriate management of high-grade precancerous lesion
Screening post-hysterectomy	ACS	Women who have had a hysterectomy (including removal of the cervix) should stop screening. Women who had a supra-cervical hysterectomy (cervix intact) should continue screening according to guidelines.
	ACOG, USPSTF	Women who have had a hysterectomy (including removal of the cervix) should not be screened anymore.
Screening among women who have received the HPV vaccine	ACS, ACOG, USPSTF	Screen according to guidelines
Screening of HIV-seropositive women	CDC	Women less than 30 years ● Screening should start within 1 year of onset of sexual activity or at the time of HIV diagnosis, but not later than 21 years old. ● Screening is done by cytology alone. Co-testing is not recommended. ● If the initial testing is negative, it should be repeated 12 months (possibly 6 months) later. ● If the results of three consecutive tests are normal then testing is done every 3 years. Women aged 30–65 years ● Screening starts at the age of HIV diagnosis if not started earlier. ● Screening is done by cytology or co-testing. ● If screening is done by cytology, the testing frequency guidelines are the same as for younger women. If cytology shows > ASC-US, refer for colposcopy. If cytology shows ASC-US, repeat it in 6–12 months. If the result is ASC-US or worse, refer to colposcopy. ● If screening is done by co-testing: - Both tests (cytology + HPV) are entirely negative; screening is repeated in 3 years. - Cytology is negative but HPV is positive (but not for HPV-16/18), screening is repeated in 1 year. If at that time either test is abnormal, refer the patient to colposcopy. - Cytology is negative and HPV is positive for types 16 or 18, refer the patient to colposcopy. - Cytology is abnormal for ASC-US and HPV positive or cytology is abnormal for >ASC-US, refer the patient to colposcopy. If the cytology is positive for ASC-US and HPV is negative, repeat cytology in 6–12 months. If the result is ASC-US or worse, refer the patient to colposcopy. Screening stops after age 65 years

[a]ACS, American Cancer Society (272); ACOG, American College of Obstetrics and Gynecology (407–409); USPSTF, U.S. Preventive Services Task Force (www.uspreventiveservicestaskforce.org/uspstf11/cervcancer/cervcancerrs.htm); CDC, Centers for Disease Control and Prevention (http://aidsinfo.nih.gov/guidelines/html/4/adult-and-adolescent-oi-prevention-and-treatment-guidelines/0) (410)

is not yet recommended by public health authorities in the United States, except in New York State, as a standard practice in the HIV-infected populations (247, 417). The United Kingdom and Australia have also ruled against this approach at present (150, 420) Any symptomatic patient should be appropriately evaluated. Annual digital rectal exams may also be helpful in detecting intra-anal masses that may be cancerous in HIV-infected individuals or in HIV-negative MSM (247).

Although male circumcision may reduce penile HPV viral load and the risk of transmission to the female partner, the evidence is inconsistent and there are no data on the effect on disease (421–428). Consequently, routine male circumcision is not presently recommended for the control of HPV diseases.

TREATMENT

A consistently effective and safe treatment for HPV infections is not available. Present therapeutic options are directed at eradicating the disease by destroying the lesions with physical or chemical means or by stimulating an inflammatory or immune response (Tables 7 to 9). A majority of these treatments have been developed empirically, but few have been thoroughly tested, and none is completely satisfactory. This section reviews the most commonly used and best-evaluated forms of treatment for HPV diseases. Additional information on these and other approaches is available (247, 429–441).

Chemical Methods

Acids

Salicylic acid alone or in combination with lactic acid (SAL) has well-established efficacy for the topical treatment of cutaneous warts (432, 436, 442). This is a keratolytic agent, but the exact mode of action is unknown. Many salicylic acid-based compounds are available as over-the-counter medications for self-treatment of cutaneous warts. They are well tolerated, although there can be hypersensitivity reactions to colophony, a component of the collodion base often used in salicylic acid preparations.

Bichloroacetic acid (BCA) and trichloroacetic acid (TCA) are keratolytic, cauterizing agents favored by gynecologists for the treatment of genital warts on moist areas (436, 441–443). They should not be applied on large areas, and their application is painful. Adverse reactions, including ulceration and scabbing, are more common than with cryotherapy. Monochloroacetic acid in combination with 60% salicylic acid has been more effective than placebo (83% versus 54%) for the treatment of plantar warts (444).

Fixatives

A 3% formaldehyde solution or 10% glutaraldehyde solution or gel is effective for the treatment of plantar warts, but sensitization to these compounds can occur. In addition, formaldehyde may cause painful skin fissures. The true efficacy of these approaches is difficult to assess in the absence of controlled trials (432, 442).

TABLE 7 Some common treatment modalities for cutaneous warts

Agent (U.S. commercial preparation)	Usual formulation and regimen[a]	Wart type[b]	Complete response rate[c] (%)
Salicylic acid (17%)-lactic acid-collodion, 1:1:4 or salicylic acid (40% or 17%)	Daily for up to 12 wk	HW	67
		Single PW	84
(e.g., Duofilm, Occlusal-HP, Wart-Off, CompoundW, Clearaway Wart Renover)		MW	50
Fixatives			
Formaldehyde, 3%	Daily (bedtime)	PW	94
Glutaraldehyde	Daily	MW	47
Podophyllin (Podocon-25 or Podofin)	15% Podophyllin, qd	Single PW	81
5-FU (Effudex cream, 5%)	2% 5-FU in propylene glycol, qd	MW	47
	5% 5-FU in DMSO, qd	MW	53
Bleomycin	1-mg/ml solution, 0.1–0.2 ml/wart	HW	~70
	Intralesional, once, 0.7% solution	MW	~50
Retinoids	0.05% Tretinoin cream	CW	85
	Etretinate, orally	CW	84
Cantharidin (Cantharone,Verr-Canth)	Topical, once	CW	87
Silver nitrate stick	qd or every 3 days	CW	43
Cryotherapy	Every 3 wk, up to 6 times	CW	41–45
		HW	~75
		PW	29–50
Electrosurgery	Once	PW	65
CO_2 laser surgery	Once	CW	32
		Periungual	71
		PW	50–90

[a] qd, once a day; DMSO, dimethyl sulfoxide.
[b] CW, cutaneous warts; HW, hand warts; PW, plantar warts; MW, mosaic warts.
[c] These rates are derived from available studies. Because of great dissimilarities between the studies and the usual absence of controls, these rates are not necessarily comparable and they are provided for indication only.

TABLE 8 Common treatment modalities for anogenital warts

Agent (U.S. commercial preparation)	Usual formulation and regimen[a]	Complete response rate[b] (%)	Relapse rate[b] (%)
Podophyllin (Podocon-25 or Podofin; 15-ml bottle)	25% in benzoin solution; qwk up to six times	35–51	60–85
Podofilox (Condylox; 3.5-ml bottle or 3.5-g tube)	0.5% Solution or gel; self-applied; bid 3 days/wk, ≤4 wk	57–72[c]	32–50
Imiquimod (Aldara; box of 12 single-use 250-mg packets; Zyclara 3.75% cream pump or box of 28 single-use 250 mg packets)	5% Cream; self-applied, tiw, qod hs, ≤16 wk	45–54	12–22
	3.75% Cream; self-applied, qd, ≤8wk	24–33	9–23
Synecatechins (Veregen; 15-g tube)	15% Ointment; self-applied, tid, ≤16 wk	50–60	3–9
BCA/TCA (Bichloracetic acid 80%, 10-ml bottle; Tri-Chlor; 15-ml bottle)	50–90% Solution; qwk up to six times	64–83	55
5-FU (Effudex cream; 25-g tube)	5% Cream; highly variable regimens	43–58	
Cryotherapy	Liquid-nitrogen spray; one or two cycles qwk up to six times	64–76	19–40
Cold-blade surgery	Scissor excision	87–94	20–31
Electrosurgery	Variable techniques (e.g., electrocoagulation, electrocautery, and fulguration)	58–94	22
CO_2 laser surgery	Variable techniques	93–99	49–65[d]

[a]qwk, once a week; bid, twice a day; tid, thrice a day; qod, every other day; hs, at night; tiw, thrice weekly; MU, million units.
[b]These numbers represent the 95% confidence limits, whenever appropriate, derived from results of selected studies.
[c]Results given for the solution formulation.
[d]Based on randomized comparative studies.

Antimitotics, Antimetabolites, and Cytotoxic, Blistering, and Cauterizing Agents

Antimitotics and antimetabolites take advantage of the increased cellular replication in wart tissues. Podophyllin, an extract from the rhizome of *Podophyllum peltatum* or the more potent *Podophyllum emodi* (436, 441, 442), is usually prepared as a benzoin tincture, 25% podophyllum (USP). Podophyllotoxin, a lignan, is the molecule most responsible for the anti-wart and toxic activities of podophyllin. It is available as a purified compound, podofilox. Podophyllin and podofilox act by preventing microtubule polymerization, thus disrupting the mitotic spindle. Podophyllin may also damage HPV DNA. These preparations have been most extensively used for the treatment of condyloma acuminatum. Local adverse reactions (pain, erythema, tenderness, erosions, and ulcerations) result from the intense acute inflammation and necrosis following topical application. Care should be exercised not to paint the healthy skin or to leave the medication on for more than 24 h. Podofilox (solution or gel) can be applied by the patient, but podophyllin should be applied by a health practitioner and typically no more than once a week (441). The efficacy of these agents is probably equivalent and superior to placebo, and the side-effects are similar (439, 441, 445). Systemic adverse reactions include sensitization to benzoin or guaiacum wood. Ingestion or extensive application of these compounds has been responsible for gastroenteric, neurologic, hematologic, and renal toxicities that may be fatal (439, 441). Podophyllin or podofilox should not be used during pregnancy. Treatment is contraindicated if the wart area is greater than 10 cm^2. Podofilox should be preferred over podophyllin because of ease of use and purity; podofilox does not contain the mutagenic flavonoids quertecin and kaempherol.

5-FU, a pyrimidine analog, prevents DNA synthesis by blocking the methylation of thymidylic acid (436, 441, 442) and is used as a 5% cream for the treatment of anogenital warts. Sensitization dermatitis and ulcerations occur infrequently but can be severe. Topical 5-FU has been associated with hematologic abnormalities, including bone marrow suppression and vaginal adenomatosis or carcinoma. The use of 5-FU is contraindicated during pregnancy. Another pyrimidine antagonist, 5-iodo-2′-deoxyuridine (idoxuridine) has been used topically for condylomata acuminata, with up to an 80% complete response rate (446, 447).

Bleomycin is a glycopeptidic mixture that causes breaks in single- and double-stranded DNA. This cytotoxic agent is effective for the intralesional treatment of cutaneous warts, particularly periungual warts (432, 436, 441, 442). At the recommended doses (<5 mg), no systemic toxicities have been reported. Most patients experience pain that can be severe. Administration of a local anesthetic or the addition of lidocaine to bleomycin helps control pain. Intralesional administration imposes a limit on the number of lesions to be treated. Alternate modes of delivery, such as bifurcated needle puncture, dermography (tattooing machine), and medicated topical dressing, have been used. Bleomycin should not be used in children, pregnant women, or patients with peripheral vascular disease.

Estradiol and 16-α-hydroxyestrone are estrogenic compounds that stimulate the growth of experimental human papillomas. Indole-3-carbinole (I3C), a molecule abundant in cruciferous vegetables (e.g., cabbage and broccoli), has an inhibitory effect by increasing the 2-hydroxylation of estradiol, thus favoring the formation of 2-hydroxyestrone instead of 16-α-hydoxyestrone (448). I3C (Indoplex) is a popular dietary supplement in the management of recurrent respiratory papillomatosis. While one study of oral I3C for the treatment of CIN 2 or 3 has shown promising results, its effectiveness for the treatment of recurrent respiratory papillomatosis has not been rigorously established (102, 103, 449).

Retinoids impede epidermal growth and differentiation. A 0.05% tretinoin cream more than doubles the complete

TABLE 9 Suggested approaches to the treatment of warts

Type of wart or lesion	Treatment options[a]
Plantar and hand warts	First line
	Salicylic acid-based paints (H)
	Second line
	Formaldehyde (H)
	Glutaraldehyde (H)
	Silver nitrate stick (H)
	Cryotherapy (O)
	Cantharidin (O)
	Bleomycin (especially periungual warts) (O)
	Third line
	Electrosurgery (hand warts only)(O)
	Laser surgery (O)
	Contact immunotherapy (O)
Flat warts	First line
	No treatment
	Second line
	Cryotherapy
	Electrocautery
Epidermodysplasia verruciformis lesions	Benign-appearing lesion
	Observation or treatment; sun protection measures
	Preinvasive or invasive malignant lesion
	Surgical excision
	Cryotherapy
	Laser surgery
Condylomata acuminata	First line
	Imiquimod (H)
	Podofilox (H)
	Veregen (H)
	Cryotherapy[b] (O)
	BCA/TCA (O)
	Scissor excision[b] (O) (if few and small lesions)
	Second line
	Electrosurgery (O)
	Laser surgery (O)
	Infrared coagulation (O)
	Third line
	5-FU (O or H)
	Photodynamic surgery (O)
Intranal warts	Cryotherapy
	BCA/TCA
	Infrared coagulator
	Cold-blade surgery
	Electrosurgery
	Laser surgery
Vaginal warts	Cryotherapy (liquid nitrogen spray, not a cryoprobe)
	TCA
	Laser surgery
Cervical warts	Electrosurgery (LEEP)
	Cryotherapy
	Laser surgery
	BCA/TCA
Intraepithelial neoplasia of the external genitalia	Laser surgery
	Cryotherapy (penis)
	Cold-blade surgery
	Imiquimod
AIN	Infrared coagulator
	BCA/TCA
	Imiquimod
	Cryotherapy
	Electrosurgery
	Cold-blade surgery
VAIN	Laser surgery
CIN	Electrosurgery (LEEP)
	Cryotherapy
	Laser surgery
Recurrent respiratory papillomatosis	Primary therapy
	Microsurgery with microdebrider
	CO_2 laser surgery
	Photodynamic (laser) therapy
	Cold-blade surgery
	Adjuvant therapy
	Intralesional cidofovir
	Interferon
	I3C
Oral warts (papillomas, verrucae, condylomata)	Cold-blade excision
	Cryotherapy
	Laser surgery
Focal epithelial hyperplasia	No treatment

[a]H, home treatment; O, office-based treatment. The reader may also consult several published guidelines or reviews mentioned in the text (references 3, 9, 16, 26, 42, 86, 88, 89, 134, 160).
[b]Recommended for intrameatal warts.

response rate compared to placebo in patients with plantar warts (442). Their efficacy in the treatment of CIN or genital warts has been limited or absent (442). Uncontrolled trials have also indicated a beneficial effect of oral or systemic retinoids for the treatment of plantar, flat, and common warts (442, 450–452). Retinoids are teratogenic.

Cantharidin is a compound extracted from the blister beetle, *Cantharis vesicatoria* (Spanish fly) (442). Its topical application causes acantholysis. Excellent responses have been documented for the treatment of cutaneous warts. The blistering is painful, but it is usually tolerable and does not cause scars. Cantharidin should not be applied on healthy skin or near mucosal membranes, especially the conjunctiva.

It is not recommended for use during pregnancy. Systemic absorption of cantharidin causes severe toxicities.

Silver nitrate sticks are chemical cauterizers that have been applied to cutaneous warts (442). Three applications at 3-day intervals on common warts resulted in a 4-fold increase in complete response compared with placebo (453). Occasional hyperpigmentation is reported. Different oral or topical preparations of zinc sulfate have been reported superior to placebo for the treatment of cutaneous warts (432, 454, 455).

Allergic Sensitization

Sensitization with 1-nitro-2,4-dinitrochlorobenzene followed by application of the compound on the lesions has been used for the treatment of recalcitrant cutaneous and genital warts (456). Complete response rates of about 80% to 100% have been reported in uncontrolled studies. The occurrence of severe allergic reactions has led to a decline in use. More recently, other sensitizing agents, 2,3-diphenylcyclopropenone (diphencyprone), squaric acid dibutyl ester, and 10% masoprocol cream (Actinex), have been advocated because of their greater safety (456).

Interferons, Immunomodulators, Antivirals, and Others

Interferons have been extensively evaluated in the treatment of condyloma acuminatum and also used to treat other HPV diseases (436, 441). Intralesional administration is more effective than placebo or parenteral IFN administration, and most studies have failed to show benefit with parenteral interferon. Recent studies suggest superiority to either placebo or podofilox for the treatment of external genital or intravaginal warts (441). When used in combination, interferon treatment adds little to the efficacy of cryotherapy or laser therapy, but it enhances the effectiveness of podophyllin (457). Topical interferon formulations are not available in the United States. Systemic interferon has little effect on HPV DNA copy number *in vivo* (457). Experience with the pegylated formulations of interferon is lacking. The common side effects of interferon are well known, and their intensity is in part related to dose. Interferons are very costly and contraindicated during pregnancy.

Imiquimod, an imidazoquinoline, is an inducer of IFN-α and other cytokines that is available as a 5% cream (Aldara) administered thrice a week or a 3.75% cream (Zyclara®) applied daily for the treatment of condyloma acuminatum (441, 442, 458, 459). An attractive feature of this immunotherapy is that it is patient applied. In patients randomized to topical treatment with imiquimod at 5% or 1% or vehicle cream every other night for up to 16 weeks (459), the complete response rates were 50%, 21%, and 11%, respectively. During a 12-week follow-up for those free of lesions, the corresponding recurrence rates were 13%, 0, and 10%, respectively. Side effects of imiquimod are local and include itching and burning sensations, as well as erythema, erosion, and swelling. Women respond approximately twice as well as men, but higher rates of disease eradication, as well as of local side effects, can be obtained with daily application (459, 460). The 3.75% imiquimod cream formulation administered daily has given a complete clearance rate of 28% compared to 9% in the vehicle arm 16 weeks after treatment initiation (461). Imiquimod has been used for the treatment of cutaneous warts, Bowen disease, and Bowenoid papulosis (459, 462, 463). It is superior to placebo or lesion ablation for the treatment of VIN (464, 465).

Cimetidine has immunomodulatory properties in addition to its H2-blocking effect. Although advocated for the treatment of cutaneous warts, it was ineffective in controlled studies (466, 467). Cidofovir [(S)-1-(3-hydroxy-2-phosphonyl-methoxypropyl) cytosine (HPMPC); Vistide], a nucleotide analog inhibitory for several DNA viruses (see Chapters 12–14), is effective in a variety of HPV diseases when delivered either intralesionally or topically (468, 469). These include condyloma acuminatum, recurrent respiratory papillomatosis, aerodigestive tumors, VIN, CIN, and cutaneous and oral warts (468–470). These results, except for condyloma acuminatum, are all based on observational studies. Cidofovir is a potential carcinogen in humans. Ribavirin, a nucleoside analog with known activity against RNA viruses, was found to have some efficacy in a cottontail rabbit papillomavirus animal model, as well as an encouraging effect in a few pilot studies on recurrent respiratory papillomatosis (102, 103, 469).

Veregen (sinecatechins or polyphenon E) is a botanical made from a partially purified fraction of a water extract of green tea (*Camellia sinensis*) leaves. It contains more than 65% epigallocatechin gallate and other catechins. The mechanism of action is unknown, but it includes cytotoxic and apoptotic properties (471, 472). It is available as a 15% ointment that is self-applied three times a day until complete lesion disappearance, but for not more than 16 weeks. In clinical trials, it showed a complete response rate of 55%, compared to 35% for the placebo (473), with better results in females than males. Erythema, itching, burning, pain or discomfort, and erosion or ulceration occur in at least about half of treated patients. The drug is contraindicated during pregnancy. The potential drawbacks of this new medication are the frequency of application—no compliance data are available—and its red color, which may stain undergarments.

Physical Methods

Curettage and Cold-Blade Excision

Curettage is used for the removal of cutaneous warts but has not been rigorously evaluated (474). Its main drawback is the formation of scars that are sometimes painful. Excision of anogenital warts with scissors has been used with good success (475, 476). Scarring is also a complication but is minimal (476). Local anesthesia is required with either technique.

Cold-blade conization of the cervix has long been the standard mode of biopsy and treatment for CIN (477, 478) but is now being replaced by electrosurgical techniques. The development of microresectors, small instruments that resemble biopsy forceps, has facilitated the resection of laryngeal papillomas.

Electrosurgery

Electrosurgery encompasses various techniques in which tissue ablation is the result of electric current. Depending on the wave form of the current, its voltage and amperage, and the number (one or two) and contact of the electrodes with the tissue, the methods are called electrocautery, electrodessication, electrofulguration, electrocoagulation, and electrosection (479). It is unknown which method is best, but scarring is a notable side effect with all. Electrosurgery should be discouraged for plantar warts, because the scars tend to be painful (480). Electrofulguration is useful for the treatment of facial warts, and electrocoagulation and electrodessication are appropriate for the treatment of condy-

loma acuminatum (441, 481). A topical anesthetic containing prilocaine and lidocaine (EMLA cream) provides good pain control for the removal of condylomata acuminata (482).

Large loop excision of the transformation zone (LLETZ), also known under the more general descriptor of loop electrosurgical excision procedure (LEEP), is an electrosurgical procedure that has largely replaced cold-blade conization of the cervix in the management of CIN (483, 484). It requires local anesthesia. It has been associated with higher rates of preterm deliveries (485).

Cryotherapy and Heat Therapy

Cryotherapy relies on four types of cryogenics: mixture of dimethyl ether and propane (DMEP) (boiling temperature −40°C), carbonic ice (−78.5°C), nitrous oxide (−89.5°C), and liquid nitrogen (−196°C) (486, 487). The treatment is delivered by a cryogenic pencil, a cotton swab, a melamine sponge, a cryoprobe, or a spray. Cryogenic pencils have been responsible for wart transmission, making liquid nitrogen spray the preferred method of delivery. The aim is to produce an ice ball around the lesion in the form of a frozen halo extending 1 to 2 mm beyond the margins. Some advocate a second freezing immediately after the lesion thaws, which may be advantageous for the treatment of plantar warts if not hand warts. Freezing is accompanied by a brief stinging sensation. Mild discomfort or pain may reappear after tissue thawing. Scarring is infrequent and usually minimal (457). The procedure is well tolerated by the vast majority of patients (457). Cryotherapy is widely used for the treatment of most HPV diseases, including CIN (484, 488). It is also the procedure of choice for pregnant patients.

Heat has been used to a lesser extent than cold to treat HPV diseases. In one study, the infrared coagulator was effective in completely eradicating condyloma acuminatum in 82% of patients (489). This technique is also becoming attractive for the treatment of AIN, including high grade, because of good clearance and control rates of the disease (490). Cold coagulation requires a 100°C probe to be in contact with the lesion for 20 s. This technique has been applied to the treatment of CIN, with success rates exceeding 93% in some uncontrolled reports (491). Anecdotal but dramatic improvements have been reported after either immersion of cutaneous warts in a 45 to 50°C water bath for up to 75 min or application of a radiofrequency heat generator for 30 to 60 s (492). In a controlled experiment, 86% of 29 treated warts disappeared, compared to 41% of 17 control warts (492). Further support comes from a randomized, patient-blinded, placebo-controlled trial in which 54% of 28 patients with plantar warts responded, compared to 12% of the 26 controls (493).

Laser Therapy

The CO2 laser has been the laser of choice for the treatment of HPV diseases, but others, including the pulsed-dye, argon, and KTP lasers, have also been used (441, 494, 495). The energy of its infrared light (λ = 10,600 nm) is well absorbed by the intracellular water, which is then vaporized. Varying the width of the beam controls the energy density of the light delivered, which can be used for cutting (narrow beam) or superficial vaporization (broad beam) (496). One can reduce the energy of a broad beam so that the tissue is coagulated rather than vaporized, a technique called brushing. An additional variable is how the light is emitted: continuously, pulsed, or superpulsed, the last being favored. The determination of proper laser techniques has been largely empirical. The great variation in the techniques used may account for differences in outcomes (441, 496–498). Because of the pain generated and the need for the patient's cooperation, laser ablation usually requires local or general anesthesia. A lidocaine/prilocaine cream (EMLA cream) is an effective solution for local anesthesia of the external genitalia (499). Postoperative pain, bleeding, swelling, and scarring occur in up to a quarter of the patients treated. The high cost of the procedure is an additional drawback. Laser therapy is a suitable option for the treatment of vulvar warts during pregnancy.

Photodynamic laser therapy is an evolving and successful approach for the treatment of cutaneous warts, genital HPV diseases, and recurrent respiratory papillomatosis (102, 103, 247, 432, 434, 436, 441, 500, 501). The concept is to deliver a photosensitizing agent to the lesion, either topically (5-aminolevulinic acid) or systemically (e.g., meso-tetra (μ-hydroxyphenyl)porphyrin (μ-THPP) or 5-aminolevulinic acid). Exposure of the tumor to intense light of the proper wavelength activates the compound and selectively destroys the tissue.

Occlusive Therapy

The therapeutic application of a poultice on a wart is a very old idea, and it has received a lot of popular attention, especially after the positive results of a randomized clinical trial in which the occlusive dressing was made of duct tape (502). This trial had limitations with blinding and follow-up, and two subsequent trials failed to replicate these results (503, 504).

Therapeutic Vaccines

Many efforts are ongoing to develop a therapeutic vaccine, but none is presently commercially available (505–507).

Suggestion, Hypnosis, and Homeopathy

The idea that cutaneous warts respond to suggestion is widely disseminated, but randomized, controlled studies have failed to provide convincing evidence of a suggestion effect (442). Hypnosis was found by some investigators to be superior to suggestion, but it appears that the results are inconsistent and the methodologies are defective (442, 508, 509). Homeopathy has so far failed to show superiority to placebo for the treatment of either plantar or cutaneous warts (432).

Management and Treatment Approaches

Our incomplete knowledge of the natural history of HPV diseases and of the comparative effectiveness and tolerance of the available therapies has unfortunately resulted in a wide variety of management options whose merits are difficult to ascertain (247, 429–436, 438–441). Furthermore, some of the treatment options are often guided by the resources available. Table 9 offers some guidelines, and the following section addresses more specific points of management.

Cutaneous Warts

In most patients cutaneous warts cause little inconvenience and resolve spontaneously in half of the patients within 1 year (206, 207), making treatment unjustified. If treatment is initiated, SAL paints have demonstrated their efficacy when compared to a placebo (432). SAL paints and fixatives can be applied by the patient and should be the first line of treatment. Cryotherapy is as efficacious as SAL (510). Contact immunotherapy and photodynamic therapy are likely to be effective treatment, but they require a skilled

practitioner and are probably better reserved for recalcitrant disease. The more traditional treatments that include bleomycin, glutaraldehyde, cantharidin, and fixatives largely remain of uncertain effectiveness (432, 442). Paring of plantar warts prior to topical treatment increases the effectiveness of the treatment. This can be done by the patient's using a pumice stone or by the practitioner with a scalpel blade. The surgical methods (cold-blade, electrosurgery, and laser surgery) should be used wisely, as they can create painful or disfiguring scars. No treatment is highly successful, the complete response rate being about 60% to 70% at 3 months (432). Therefore, it is important to avoid the production of scars and to know when to withhold treatment. It is well established that plantar warts, especially mosaic warts, are more difficult to eradicate than common warts. Flat warts are the easiest to eliminate.

Epidermodysplasia verruciformis lesions should be monitored for premalignant and malignant transformation as well as protected from sun exposure (246, 511). Excisional surgery, cryotherapy, or laser surgery can be used for the management of the lesions (246, 511). Medical treatments have also been applied, including with interferon and retinoids (246, 512). Grafting may be necessary to cover extensive skin defects, and artificial skin has been used successfully to that effect.

Anogenital Lesions

Condyloma Acuminatum

Treatment for condyloma acuminatum is aimed at improving cosmesis, relieving symptoms, or freeing an obstructed birth canal. It is also directed at the often seriously compromised psychological well-being of the patient (513–518). The possible impact of treatment on HPV transmission, development of an HPV-related malignancy, or on the acquisition of recurrent respiratory papillomatosis in the newborn are uncertain (114, 205). Presently, treatment should not seek to eradicate subclinical HPV infection; such attempts have failed and caused high morbidity (193). The benefits of treating lesions should be weighed against adverse effects and costs associated with therapy and against the possibility of spontaneous resolution, which occurs within 3 to 4 months in approximately 10% to 20% of patients. Other than immunosuppression or immunodeficiency, with the possible exception of long disease duration, there are no strong or reliable indicators of disease refractoriness to treatment (457). Warts appearing at new sites (recurrence) or during treatment can be treated with the same therapy. Warts appearing at previously treated sites (relapse) may benefit from a different therapy.

There is no evidence that evaluating and treating the male partner has an impact on HPV infection, CIN risk, or disease relapse in the treated woman (193, 247, 438). However, no comparable data exist regarding the female partner of the male patient. Nevertheless, it is appropriate to evaluate the sexual partner for three reasons: (a) a chance to address specific STD transmission risks; (b) the possibility to lighten the patient's psychosexual burden and reassure about the partner's health status; and (c) the offer of education and counseling (247). Condom use can be beneficial in couples in which at least one of the partners has HPV disease and should be advocated during the treatment period (see "Prevention" section).

Podophyllin is inferior to podofilox, excisional surgery, electrosurgery, and cryotherapy (247, 441, 519). Although podofilox is more effective than podophyllin, both drugs are associated with high recurrence rates. Podofilox is superior to a vehicle only in preventing recurrences when used prophylactically after podofilox treatment. Cryotherapy and TCA application appear to be therapeutically equivalent, and cryotherapy is not significantly different from electrosurgery (519).

Self-treatment options offer convenience and privacy. Imiquimod and podofilox are attractive, the former being likely more effective if one takes into account the relapse rate. Veregen is more costly, requires three applications a day, and stains the skin and clothing. The downside of self-treatment is the long duration and frequency of treatment. A practitioner can offer possibly more effective and definitely faster solutions with cryotherapy and TCA application. Electrosurgery (and that would include the infrared coagulator) and laser surgery require both equipment and skills. Surgical excision with scissors is well suited to the treatment of lesions that are small in size and number. Urethral warts can be treated with cryotherapy or surgical excision (247). Laser surgery should not be first-line choice because of high cost. For recalcitrant lesions laser surgery is useful, especially if the lesions are numerous. Other options to consider are higher frequency of administration of imiquimod, intralesional interferon, photodynamic therapy, allergic sensitization, 5-FU application, or cidofovir. Lesions in moist areas appear to resolve better than ones on dry areas but not necessarily because of the treatment received (520–522). Furthermore, warts located within the occluded foreskin have a favorable outcome (523).

Cryotherapy, BCA/TCA application, electrosurgery, cold-blade excision, and laser surgery are all appropriate for the treatment of condyloma acuminatum in the pregnant patient. Anogenital warts in immunocompromised patients respond relatively poorly to treatment and a have a high rate of relapse (524, 525). Nevertheless, some patients do respond and there are few reasons to withhold treatment in these patients, if otherwise indicated. The efficacy of imiquimod is reduced by half in HIV-seropositive patients when comparing then to HIV-seronegative patients, but it remains a useful agent to control disease (often the main goal in these patients) and recurrences (526, 527). It is uncertain whether HAART affects the natural history of genital warts, oral warts, CIN, or AIN (144, 528–538). From a practical standpoint, it is unlikely to have a significant effect on the treatment decisions related to HIV and HPV.

No intervention, either as a primary approach or because of previous treatment failures, may be necessary in the absence of changing lesions, intraepithelial neoplasia, or carcinoma, provided proper follow-up and evaluation (e.g., yearly) are done.

Internal Warts and Intraepithelial Neoplasias

The evaluation and management of intraepithelial neoplasias and internal warts should be left to experienced practitioners (247, 484, 494, 539–543). Guidelines have been established for the cervix (http://www.asccp.org/Guidelines-2/Management-Guidelines-2) (267).

Patient Education

The public is largely uninformed about genital HPV disease, and the information is complex; thus, education is a necessary part of the practitioner-patient interaction. The diagnosis of genital HPV disease is emotionally charged, and education is also an opportunity to engage the trust of the patient so that optimal care can be offered. For patients and health professionals, abundant high-quality and current

information materials (including in Spanish) about HPV are easily available from the Centers for Diseases Control and Prevention (CDC) (http://www.cdc.gov/hpv/ and http://npin.cdc.gov/). The CDC offers a hotline 1-800-CDC-INFO (232-4636), TTY: 1-888-232-6348 (Monday through Friday from 8:00 a.m. to 8:00 p.m., EST), e-mail: cdcinfo@cdc.gov. The American Social Health Association (http://www.ashastd.org) also offers excellent resources for patients, including links to support groups. It also offers person-to-person counselling that can be arranged on the web site (http://www.ashasexualhealth.org/person2person-2/).

Recurrent Respiratory Papillomatosis

Although numerous therapeutic options have been considered for the treatment of recurrent respiratory papillomatosis, the microdebrider device is now favored by most American surgeons instead of the CO2 laser or cold-steel surgery (103, 544). Photodynamic laser therapy will probably gain acceptance due to early encouraging results. Because many patients require scores of procedures during a lifetime, selection of a skilled operator is essential to minimize long-term side effects. Tracheostomy should be avoided since the surgical site often becomes involved with the disease, which may also spread further down the respiratory tree. Radiotherapy of recurrent respiratory papillomatosis has been associated with malignant transformation and is contraindicated. Parenteral interferon is used as an adjuvant therapy. Another adjuvant treatment, intralesional cidofovir, has now replaced oral ribavirin (544, 545). Most patients will have tried I3C (Indoplex). Patients and families may find support and information at the Recurrent Respiratory Papillomatosis Foundation (http://www.rrpf.org), or the International RRP ISA Center (http://www.rrpwebsite.org).

Oral Warts

Surgical excision and cryotherapy application are among the therapeutic choices for the management of oral warts (62, 258). Focal epithelial hyperplasia usually does not require treatment because of its self-limited evolution.

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Human Parvoviruses

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Parvoviruses have been isolated from a wide range of animals, including mammals, birds, insects, crustaceans, and reptiles. These viruses tend to be species-specific and can cause a variety of serious diseases in their host species (1). The first parvoviruses isolated from humans were adeno-associated parvoviruses, which have not yet been linked with disease. Until recently the only parvovirus associated with human disease was human parvovirus B19 (B19V), which was fortuitously identified in 1975 during an evaluation of tests for hepatitis B virus antigens (2). B19V has been associated with erythema infectiosum, transient aplastic crisis, chronic anemia in patients with impaired immune systems, hydrops fetalis, and purportedly a number of other conditions (3). Seven additional parvoviruses have recently been detected in humans by molecular screening for new sequences, including human bocavirus (HBoV)1–4, tetraparvovirus (PARV4), bufavirus (BuV), and tusavirus (TuV) (4–9). HBoV1 causes acute respiratory illness (10) and, as with HBoV2 and 3, possibly also encephalitis (11). The other recently discovered human parvoviruses are yet to be associated with human disease.

VIROLOGY

Classification and Genotypes

Parvoviruses are small, nonenveloped, single-stranded DNA viruses that infect a variety of animals, usually in a species-specific fashion. The family *Parvoviridae* is divided into two subfamilies: the *Densovirinae*, which infect arthropods, and the *Parvovirinae*, which infect vertebrates. The members of the *Parvovirinae* that infect humans will be the focus of this chapter. The subfamily *Parvovirinae* is divided into eight genera: *Protoparvovirus*, *Erythroparvovirus*, *Dependoparvovirus*, *Amdoparvovirus*, *Bocaparvovirus*, *Aveparvovirus*, *Copiparvovirus*, and *Tetraparvovirus* (1). The parvoviruses known to infect humans include B19V in the genus *Erythroparvovirus*, adenovirus-associated viruses (AAVs) in the genus *Dependoparvovirus*, HBoV1–4 in the genus *Bocaparvovirus*, PARV4 in the genus *Tetraparvovirus*, and BuV and potentially TuV in the genus *Protoparvovirus* (Fig. 1).

AAVs in the *Dependoparvovirus* genus require co-infection with another virus, usually an adenovirus or herpesvirus, to replicate. Multiple AAV genotypes have been identified to date, detected as co-infections of adenovirus laboratory stocks or isolated from human or nonhuman primates (12). AAVs can establish a latent infection by integrating into the host-cell genome or being maintained in an extrachromosomal state. The ability of DNA of some AAVs to integrate into the human genome has made them a highly investigated vector for gene therapy (13). Latent AAV can be rescued by infection with a helper virus or *in vitro* by stressing the cell with ultraviolet or ionizing radiation. Members of the other genera in the subfamily *Parvovirinae* do not require a helper virus for efficient productive infection but do usually require actively dividing cells. B19V belongs to the species *Primate erythroparvovirus* 1, consisting of three genotypes, 1 to 3, which all belong to one serotype (1, 14–18). Among the three genotypes, genotype 1 is presently most commonly detected overall. Genotype 2 has been frequently detected within tissues of subjects born before 1972 (19) but very seldom in blood after the 1960s, and genotype 3 has been infrequently detected with the exception of a study in Ghana (20). The three genotypes appear to have similar biologic and antigenic properties (14). Sequence studies of PARV4 have also identified three genotypes; genotypes 1 and 2 (formerly PARV5) are found mainly among injecting drug users in Western countries, and genotype 3 has been detected only in sub-Saharan Africa (21–24), where it is endemic. PARV4 belongs to the species *Primate tetraparvovirus 1* (1). To date, sequence studies of HBoV isolates have identified 4 viruses, named HBoV1–4 (4, 5, 7, 25), of which HBoV1 is a respiratory virus, whereas the others seem to be enteric. HBoV1 and 3, as well as a gorilla bocavirus (26), belong to the species *Primate bocaparvovirus 1*, whereas HBoV2 and 4 belong to *Primate bocaparvovirus 2* (1). Of the more recently discovered bufavirus, there are three genotypes with yet unknown epidemiological, biological, or clinical differences, and they belong to the species *Primate protoparvovirus* 1 (1, 8, 27).

Composition

Parvoviruses are icosahedral particles between 20 and 25 nm in diameter containing 60 copies of the structural proteins (Fig. 2). B19V and PARV4 capsids contain 2 proteins, termed VP1 and VP2, whereas AAV and HBoV capsids contain 3 proteins, designated VP1, VP2, and VP3 (28, 29). X-ray crystallography has been used to determine the structure for a number of parvoviruses including canine parvovirus (CPV); minute virus of mice (MVM); AAV2, 4, and 5; B19V;

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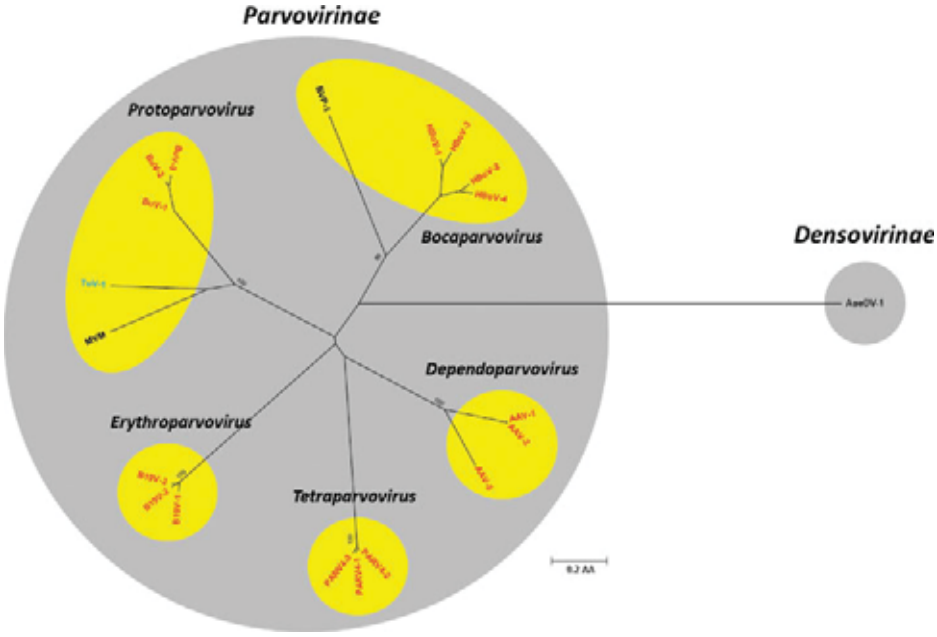

FIGURE 1 Neighbor joining phylogenetic tree derived from ORF1 (nonstructural protein) amino acid sequences obtained from GenBank and used by ICTV to classify parvoviruses (1). Subfamilies *Parvovirinae* and *Densovirinae* shown as gray circles. For simplicity, only one representative member of the *Densovirinae* is shown and only genera of the *Parvovirinae* with members known to infect humans are shown as yellow circles (*Copiparvovirus*, *Aviparvovirus*, and *Amdoparvovirus*, genera are therefore excluded). Representative human parvoviruses (in red), putative human viruses (in blue), and prototype viruses when not human (in black) are shown within each genus. AA, amino acid; AaeDV1, *Aedes aegypti* densovirus 1; AAV1, AAV2, AAV5, adeno-associated viruses 1, 2, 5; B19V1–3, parvovirus B19 genotypes 1–3; BPV1, bovine parvovirus 1; BuV1–3, bufavirus genotypes 1–3; HBoV1–4, human bocavirueses 1–4; MVM, minute virus of mice; PARV4 1–3, tetraparvovirus genotypes 1–3; TuV1, tusavirus genotype 1.

and HBoV1 (30). Common structural features among parvoviruses include one or more protrusions at the 3-fold axis, a depression at the 2-fold axis, and a channel at the 5-fold axis surrounded by a canyon. Depending on the extent of the 3-fold protrusion, the capsids appear either smooth, as for B19 and HBoV, or rough, as for AAV. Moreover, the 5-fold channel has been proposed to be a portal for genome packaging and externalization of VP1u; however, it seems to be closed in the B19V capsid. For some parvoviruses, the locations of amino acids associated with cell receptor and antibody-binding sites have been determined.

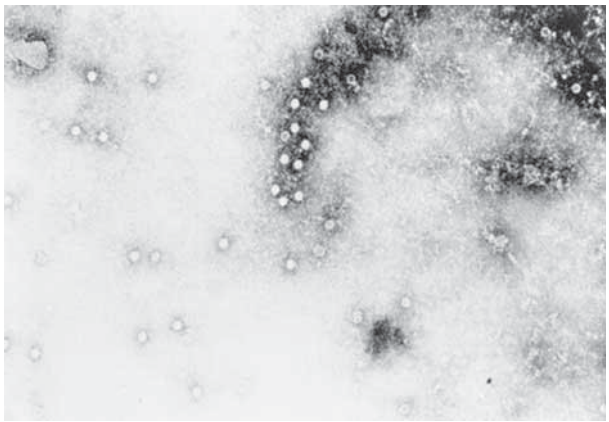

FIGURE 2 Electron micrograph (X170,000) of B19V empty particles in a serum specimen from a patient with transient aplastic crisis. Courtesy of G. William Gary, CDC, Atlanta, GA.

The encapsidated single-stranded DNA genome varies from 4 to 6 kb in size, and each virion contains either the negative or positive strand in varying proportions. AAV and B19V, and presumably PARV4, virions contain equal numbers of positive- and negative-sense DNA strands, while HBoV encapsidates mostly the negative strand (21, 31–33). Parvovirus DNA contains palindromic inverted repeat sequences at each end that form terminal hairpin structures, which can be either semi-identical or different depending on the virus. These hairpin structures permit self-priming and are essential for replication. Parvovirus genomes contain generally two major open reading frames (ORFs), one that encodes nonstructural proteins in the left half of the genome and the other in the right half of the genome for the structural capsid proteins. HBoV, as with the other bocaviruses, has an additional minor open reading frame in the middle (4).

Multiple mRNA species are produced by splicing mechanisms. For example, the B19V genome encodes nine mRNA species from a single promoter (Fig. 3) (34). These 9 mRNAs translate at least 5 proteins, VP1, VP2, and at least 3 nonstructural (NS) proteins (Table 1) (35, 36, 37). The NS proteins play a key role in production of infectious virus, probably by regulating transcription, participating in replication, and assisting in encapsidation of virion DNA (38). The NS proteins can be toxic to cells, and this toxicity may contribute to cytopathology during infection. Similar to B19V, HBoV has only 1 promoter (39), whereas AAV has 3 (1).

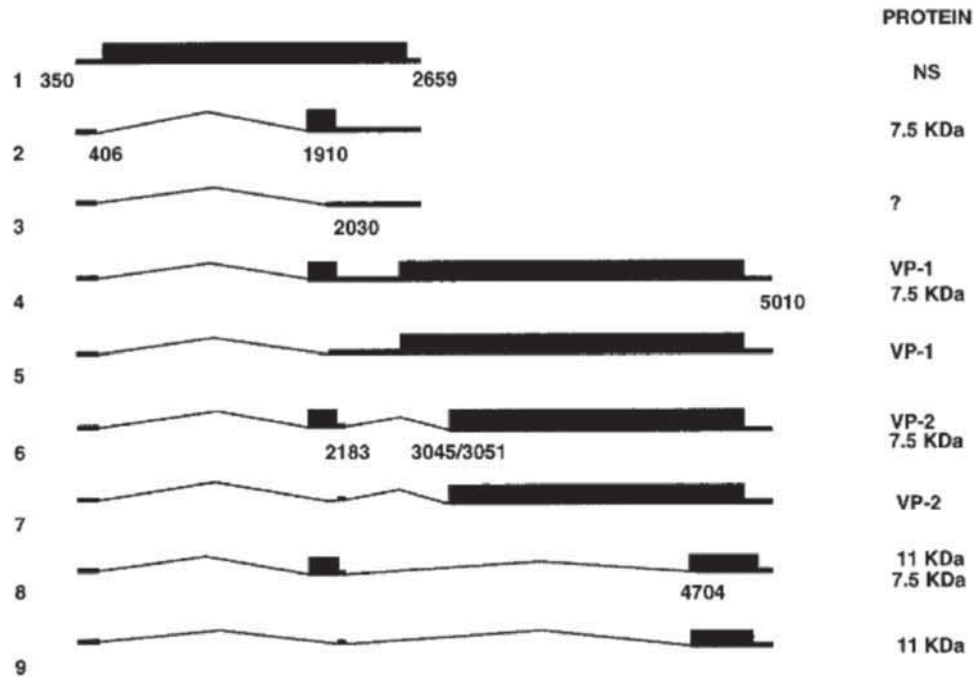

FIGURE 3 Schematic of B19 mRNA produced in erythroid progenitor cells. The thin line represents introns and the heavy line represents the nontranslated portion of exons. The filled box represents translated portions of exons. The numbers below the line indicate the start or end of the respective exon. All mRNAs are initiated at the same promotor (P6). The NS protein (first mRNA) is in one reading frame; the 7.5 kDa, VP1, and VP2 proteins are in a second reading frame (-1 relative to the NS protein); and the 11.0 kDa protein is in the third reading frame. Based on data from references 34 and 35.

VP2 or VP3 is the major component of the parvovirus virion. In the case of B19V, VP2 comprises about 95% of the virion and VP1, 5% (40). The VP2 proteins expressed in a Chinese hamster ovary-cell line or in a baculovirus system will self-assemble into empty capsids. VP1 can be incorporated into VP2 empty capsids, but VP1 alone does not self-assemble unless its unique amino terminus is truncated (41). VP1 contains the VP2 sequences plus an additional 227 amino acids unique to VP1 at its amino terminus termed VP1u. Empty capsids that include both VP1 and VP2 elicit neutralizing antibodies, while those containing only VP2 do not (42). Most parvoviruses, including all human parvoviruses, have a calcium-dependent phospholipase A2 (PLA2) motif in VP1u (43, 44). PLA2 is presumed to facilitate infection in cells by altering membranes of virion-containing endosomes.

Receptors

B19V attaches to a specific cell-surface receptor, the glycosphingolipid ceramide globoside, also called blood group P antigen (45), where it undergoes endocytosis and migrates to the nucleus. Although globoside is necessary for infection, it is not sufficient, and α5β1 integrin and KU80 autoantigen have been reported to act as coreceptors for B19V (46–48). VP1u also binds to cells and is important in determining tropism, although its receptor has not been identified to date (49). A variety of receptors have been identified for other parvoviruses, including transferrin receptors for some members of the genus *Protoparvovirus* and heparin sulfate proteoglycan, human fibroblast growth factor receptor 1, and integrin for AAVs (30, 43). The receptors for HBoVs, BuV, and PARV4 have not yet been identified.

TABLE 1 Proteins of human parvovirus B19

Protein	Size (kDa)	No. of amino acids	Function
NS protein	77	671	Replication
VP1	83	781	Virus capsid
VP2	58	554	Virus capsid
7.5 kDa	7.5	72	Unknown
11 kDa	11	94	Unknown

Replication Strategy

Efficient replication of parvoviruses requires cellular functions expressed only during the S phase of cell division and thus requires actively dividing cells. However, to ensure efficient replication, parvoviruses may utilize the cellular DNA damage response (DDR) and hypoxic conditions (50–54). Transcription of mRNA and replication of virion DNA occur in the nucleus. Replication of the single-stranded virion DNA is initiated at the terminal hairpins and produces dimer and dimer-duplex intermediate concatemeric replicative forms, involving rearrangement of the hairpins and cleavage to form single-stranded virion DNA in a process termed "rolling hairpin replication" (55). For B19V, PARV4, and AAV, the plus- and minus-sense single-stranded DNA genomes are encapsidated with equal frequency, but for bocaviruses the minus strand is favored in 85 to 95% of the capsids (21, 31–33, 56). Induction of cell apoptosis, exocytosis, or cytolysis results in virus release.

Host Range

Parvoviruses are normally host specific, i.e., the human parvoviruses infect only humans, so there are no direct animal

models for these viruses. However, serologic tests have suggested that exposed animal handlers may have been infected with one of the simian erythroviruses (57). Close relatives of B19V, HBoV, PARV4, and BuVs have been detected in animals, besides nonhuman primates, and also in domestic animals, rodents, and bats (1, 26, 58, 59). Infection of other erythroviruses in their respective host species has been proposed as a model for B19V disease (60, 61).

Growth in Cell Culture

Productive B19V propagation *in vitro* was first accomplished in late erythroid-precursor cells present in bone-marrow explant culture systems (62). Subsequently, several human leukemic cell lines, including UT-7/Epo, MB-02, JK-1, and KU812, have been shown to support low levels of B19V replication (63, 64), although CD36 cells from bone marrow remain the most efficient. As shown for several viruses, hypoxia can improve replication (65). Actively replicating cells are required for efficient replication, although B19V has also been shown to replicate in otherwise nonpermissive cells with the adenovirus providing helper functions (66).

With similar appropriate helper viruses, AAVs can replicate in a variety of tissue culture systems (67). If a helper virus is not present, AAV integrates into the host cell genomic DNA in a site-specific fashion. When cells containing integrated AAV are infected with helper virus or are subjected to certain chemical or physical treatments, AAV genes are expressed, the integrated DNA is released, and virions are produced. HBoV1 has been shown to replicate and produce progeny virions in polarized human-airway epithelial cells (HAE) (68, 69); however, it did not replicate in monolayer cultures of the same nondifferented cells. No tissue culture system has yet been shown to support PARV4, BuV, or TuV replication.

Inactivation by Physical and Chemical Agents

Nonenveloped viruses like parvoviruses are stable under ordinary environmental conditions and are more difficult to inactivate on environmental surfaces. The choice and concentration of the inactivating agent, inactivation time, and presence of organic material all impact the effectiveness of inactivation. For surface inactivation, detergent and phenolic acid-based products are generally ineffective for nonenveloped viruses, whereas 5,000 ppm of free chlorine (e.g., 1/10 dilution of household bleach) and 70% aqueous alcohol solutions inactivate some nonenveloped viruses, and 2% glutaraldehyde solutions inactivate most (70). In transfusion medicine, inactivation of blood or blood products is problematic. Infection in persons receiving heat-treated clotting-factor concentrates indicates that the virus can withstand dry-heat treatment at 80°C for as long as 72 hours or at 100°C for 30 min (71). Based on *in vitro* studies, B19V is more sensitive to liquid heat treatment, and the level of sensitivity depends on the composition of the solution (72).

EPIDEMIOLOGY

Distribution and Geography

B19V infection is common throughout the world, although the percentage of persons positive for immunoglobulin G (IgG) antibody, as a measure of past infection, varies by location and timing of the last B19V epidemic (73–75). AAVs, HBoV, and PARV4 have been detected in multiple locations globally, whereas BuV has thus far been found only in a few countries in Africa, Asia, and Europe (8, 27, 76–78). The seroprevalence of BuV is low, at least in Northern Europe (Väisänen, personal communication).

Incidence and Prevalence

B19V

In most communities, the prevalence of B19V antibody increases from 2% to 15% among children 1 to 5 years of age, to 20% to 40% among children 6 to 9 years of age, and to 35% to 60% among children 11 to 19 years of age (79). Antibody prevalence continues to increase with age, reaching 75% to 90% in several studies among persons older than 50 years of age (Fig. 4). In most countries, the greatest rate of increase in IgG positivity occurs in school-age children, consistent with the observation that erythema infectiosum, the most commonly recognized manifestation of B19V infection, is most often diagnosed in this age group, and that children are the source of most infections (80).

The seroprevalence of B19V is significantly higher in some parts of the world, including parts of Africa and Papua New Guinea, with >80% of 10-year-olds having detectable B19V antibody (81, 82). In contrast, some parts of Asia, and among isolated tribes of Africa and South America, the seroprevalence can be significantly lower (83–85).

Subclinical infection is common, and most adults with B19V IgG antibody will not give a history suggestive of a B19V-associated illness. In outbreaks of B19V infection, 50% or more of infected persons may be asymptomatic or report a nonrash illness (86–88).

B19V infections are most often noted as outbreaks of erythema infectiosum in schools. Outbreaks in the United States and Europe usually occur in the winter and spring and often continue for months or until school recesses for the summer (86, 89). During school outbreaks, 25% to 50% of students have been reported to have clinical or serologic evidence of infection. Staff in schools are also commonly infected (80, 89–91); 20% of susceptible staff developed infection during one outbreak. Infections also occur endemically when no outbreak activity is noted in the community. Since recurrences of erythema infectiosum or transient aplastic crisis are rare, it is assumed that past infection, as indicated by presence of B19V IgG antibodies, confers long-term protection from disease. Experimental inoculation of one patient with low levels of B19V IgG antibody led to an asymptomatic infection, indicating that past infection may

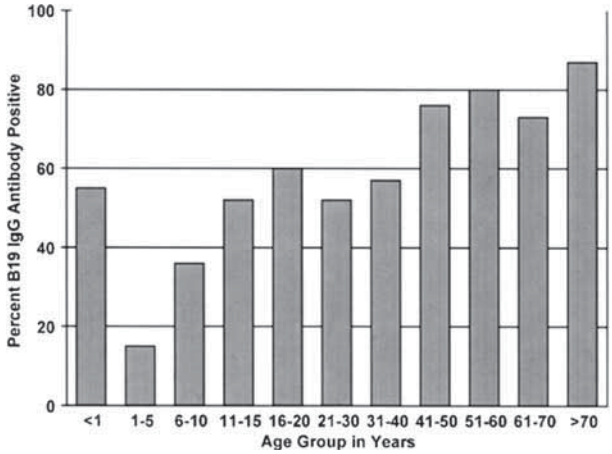

FIGURE 4 Rates of B19V IgG antibody positivity by age. Adapted from reference 73.

not always prevent re-infection but probably protects from disease (92).

HBoV and Other Parvoviruses

HBoV infections are common, with respective seroprevalences of HBoV1–4 in 6-year-olds of 80%, 50%, 10%, and 0% (93). However, serologic studies of these four HBoVs is complicated by cross-reactivity and most likely by the immunological phenomenon known as "original antigenic sin," leading to underestimates of the true prevalences (93–95). The majority of AAV data are for AAV2 for which 50% or more of persons 10 to 19 years of age have antibodies (96). Less is known about timing and extent of infection with PARV4. In European and North American populations, PARV4 infections are rare outside of persons with risk factors for blood-borne viruses, where seropositivity among the latter can exceed 90% (97–99). In contrast, studies in some sub-Saharan African and Asian countries have found a higher PARV4 seroprevalence in the absence of these risk factors (22, 24, 100, 101).

Bufavirus DNA has been detected in stools of children and adults having diarrhea, with low (0.2 to 4%) prevalence (8, 27, 78, 102) and tusavirus DNA in the diarrheal stool of one single child in Tunisia (9).

Seasonality

In temperate climates in the Northern Hemisphere, B19V outbreaks most often occur during the late winter and spring (103). These outbreaks are cyclical, with increased B19V transmission occurring every 4 to 10 years in a given community (104, 105). Seasonal patterns of AAVs and PARV4 infections are not known, and those for HBoVs are yet to be clearly defined. HBoV1 can be detected throughout the year (106–112).

Transmission

The primary route of B19V is through the respiratory tract because its DNA can transiently be found in secretions from the nose and throat (86, 87, 92). In volunteer studies, infection occurred in four of five exposed persons after intranasal inoculation (92, 113). Transmission also occurs by transfusion of contaminated blood (114, 115), from blood products including albumin and clotting factors (71, 116), from an infected mother to her fetus, and, potentially, even after tattooing (117). Depending on the community and antibody assay, between 40% and 60% of young adults will test positive for B19V IgG antibodies and thus presumably not be susceptible to infection.

The risk of B19V transmission from a single unit of blood is low, although high concentrations ($\geq 10^5$ IU) of B19V DNA were present per ml in 1/500 to 1/10,000 units in one study (118). The risk has been higher with use of many types of multi-unit blood products. B19V DNA has been detected in 25% to 100% of products, such as albumin and factor VIII concentrates, which may contain components from 5,000 or more units, and infection has often been associated with receipt of these products. The small size of the B19 virion and the resistance of the virus to inactivation have made it difficult to remove or inactivate during the preparation of these products, and, in Europe and USA, all plasma products are now required to be tested to ensure that the B19V viral load is $< 10^4$ IU/ml (119, 120).

Nosocomial transmission of B19V among staff and patients has been reported with infection rates as high as 50%, but it is often difficult to differentiate hospital-acquired from community-acquired infection in such outbreaks, except in patients with very high virus loads, like those with transient aplastic crisis (121–125).

B19V is transmitted efficiently in households, with about 50% of susceptible exposed household members becoming infected (86, 87). Infection also occurs in about 20% of susceptible staff working in a school during an outbreak of erythema infectiosum (89, 91), in about 6% of susceptible residents of a community during one B19V outbreak (88), and in about 1% to 3% of women with no identifiable exposure during a year or a pregnancy (74, 126–128). In pregnant women living in communities with outbreaks of B19V disease, infection rates among susceptible persons have ranged from 4% to 14%. Risk factors for infection include working as a teacher or childcare worker or having children at home (74, 90, 91, 129).

In a recent study of 3,710 pregnant mothers, a clear increased risk (adjusted hazard ratio, aHR=2.63; 95% CI: 1.27 to 5.46) of B19V infection among daycare employees was observed, compared with those of socioeconomically similar health care professionals (129). The difference in the infection risk was even more pronounced in the analysis of nulliparous women (aHR=5.59; 95% CI: 1.40 to 22.4), eliminating the effect of the women's own children. Vertical transmission from mother to fetus has been reported in 25% to 50% of infants of mothers infected during pregnancy (130–132).

The route of transmission for HBoV1 is the respiratory tract, but those for AAV, HBoV2–4, PARV4, BuV, and TuV are not well understood. The presence of HBoV2 and 3, as well as BuV and TuV, in feces suggests fecal-oral transmission, while HBoV4 is too rare for any conclusions (9, 78). The potential for maternal transmission of HBoV1 and PARV4 in newborns has also been documented (133, 134). The high prevalence of genotypes 1 and 2 of PARV4 in injecting drug users and persons infected with HIV, suggests that they are transmitted parenterally by contaminated needles or other blood contact (135). However, increasing evidence from regions in sub-Saharan Africa and Asia, with higher PARV4 seropositivity rates and demonstrable detection of PARV4 genotype 3 DNA in nasal and fecal specimens from children, also suggests a potential role for fecal-oral transmission (22, 24, 100, 136).

PATHOGENESIS IN HUMANS

Nucleated erythroid-precursor cells are the primary cells and bone marrow the primary tissue for B19V replication (62, 137). Extramedullary hematopoiesis makes fetal liver an important site of fetal infection *in utero* (138). Giant pronormoblasts, erythroid cells with marginated chromatin and nuclear inclusions (Fig. 5), are typical of B19V infection and are found during active infection in bone marrow, circulating blood, liver, and other fetal tissues (139).

Globoside can also be found on other cell types apart from erythroid precursors (140). B19V can be detected in these nonerythroid tissues and cells (e.g., endothelial cells of the myocardium, epidermal cells, granulocyte precursors, megakaryocytes, and macrophages) and may persist in the joints, skin, heart, liver, and even in the brain for decades following the acute infection (138, 141–145). The mechanisms for B19 genome persistence in these diverse tissues are unknown. Studies have also demonstrated the presence of B19V-like particles or B19V DNA in fetal myocardial cells and macrophages of infected fetuses (132, 146). Although replication may occur in these cell types, it appears to be relatively inefficient.

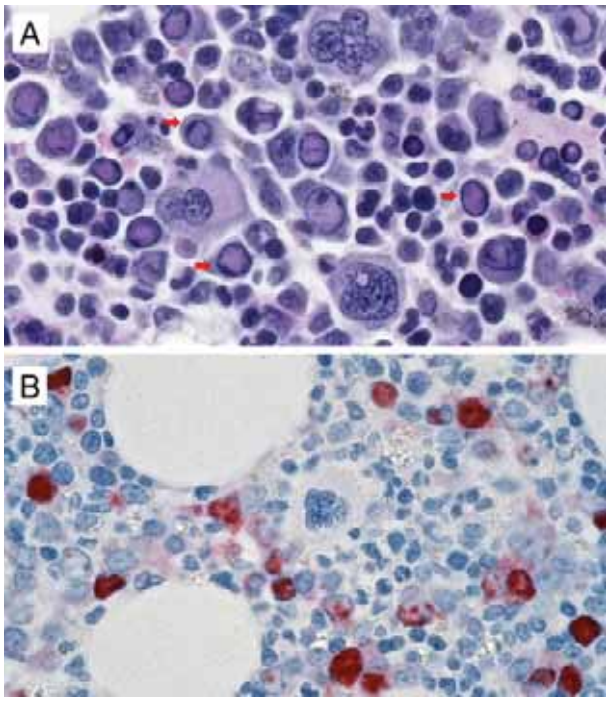

FIGURE 5 (A) Hematoxylin and eosin stain of bone marrow from patient with B19V infection. Note prominent intranuclear inclusions (arrows). (B) Immunostaining of B19V antigens in bone marrow (immunoalkaline phosphatase stain, naphthol fast red substrate with light hematoxylin counterstain). Original magnification of both images, X630. Panels courtesy of Wun-Ju Shieh, CDC, Atlanta, GA.

The course of erythema infectiosum, the most common manifestation of B19V infection, suggests disease is, in part, immune-mediated. The estimated incubation period to onset of rash is usually between 1 and 2 weeks but sometimes as long as 3 weeks (147). In experimental infection in humans, the first symptoms of fever, malaise, and myalgia occurred 1 to 2 weeks after inoculation of the virus. These symptoms developed during high viremia and were accompanied by a reticulocytopenia, drop in hemoglobin, and moderate, but clinically insignificant, lymphopenia, thrombocytopenia, and neutropenia (92). The viremia and reticulocytopenia resolved by days 11 to 16 as volunteers mounted an antibody response; the rash or arthralgias were noted at 15 to 17 days after inoculation (Fig. 6).

During the high level viremia, the bone marrow has a marked decrease in the number of erythroid cells but a relatively normal number of myeloid cells. The arrest of hematopoiesis during viremia is consistent with the expected impact of a lytic infection of red-cell precursor cells. On the other hand, the fact that onset of rash and arthritic symptoms occurs coincident with the host antibody response and decreasing levels of viremia suggests that the host immune response is important to the pathogenesis of these symptoms. The occurrence of rash in an immunocompromised patient after receiving immune globulin, and again after the patient mounted an antibody response, support this conclusion (148). B19V-antibody immune complexes have been detected in serum of experimentally infected adults by an immune adherence assay (92) and in serum of 31 of 38 B19V-infected patients by a C1q binding assay (122). The finding of B19V antigens and viral particles in vascular endothelial cells from B19V-associated rash lesions suggests that infection of these cells might also contribute to the pathogenesis of the rash (145).

The pathogenesis of B19V-associated arthritis is less clear. B19V DNA has been detected by PCR assays in synovial tissue from the joints of patients with B19V-associated arthritis, other types of arthritis, and from control patients (149–151). However, there is no evidence that B19V replicates in synovial tissue. In B19V-associated arthritis, it is possible that virally expressed proteins are cytotoxic or contribute to joint inflammation. The PLA2 activity in VP1 can activate synoviocytes, induce mediators of inflammation, and, possibly, participate in B19V-associated arthritis (152).

Less is known about the tissue tropism and pathogenesis of the other human parvoviruses. HBoV2–4 have been found mostly in stool, whereas HBoV1 has been most often detected in respiratory specimens but also in stool and serum (10, 153), as well as in adenotonsillar tissues obtained from otherwise healthy children (154, 155).

HBoV1 has been shown to infect and propagate in polarized human airway epithelial (HAE) cells in an air-liquid interface (ALI) culture (68, 69). Progeny virus particles were shown to be released from both the apical and basolateral surfaces of the HAE-ALI, while showing impaired epithelial integrity: i.e., thinning of the epithelium, decreased polarization, disruption of tight junctions, cell hypertrophy, and loss of cilia, all markers of respiratory tract injury (69). Persistent replication in these terminally differentiated cells was surprisingly observed for 50 days (156). The ability to replicate is thus not cell-cycle dependent as has generally been believed for parvoviruses. In these nondividing cells, it has been shown that HBoV1 utilizes the DNA-repair polymerases required, by the cellular DNA damage and repair (DDR) machinery, for its replication (54). Finally, by studying bronchoalveolar lavage samples of HBoV1-infected patients, an upregulation of various cytokines has been observed, similar to those seen in washes of HBoV1-infected HAE-ALI cell cultures (157). HBoV1 infection thus seems to trigger specific cytokines that may affect pathogenesis.

AAV has been most often detected in specimens from the genital tract, but it has also been detected in muscle, brain, and a variety of other tissues without causing any damage (12, 13, 142, 158). PARV4 has been detected in serum and blood specimens from otherwise healthy individuals and from autopsy bone marrow and lymphoid tissue specimens from human immunodeficiency virus–infected persons, likewise with nondefined pathology (21, 99, 106).

Virus Replication Patterns

As noted above, the B19V-associated anemia correlates with viremia and is presumed to be related to the cytopathic effect of the virus on erythropoietic precursor cells. In immunocompetent persons, the immune response controls the infection within 7 to 10 days and before a significant anemia develops. In persons with borderline compensated hemoglobin levels, even the short cessation of red cell production can produce a self-limited, but serious, anemia (i.e., transient aplastic crisis). A variety of conditions, including sickle cell disease, hereditary spherocytosis, thalassemia, and acquired hemolytic anemias, have been associated with transient aplastic crisis.

Some patients with compromised immunity due to HIV infection, malignancies, chemotherapy, or organ transplantation are unable to control B19V replication and develop a

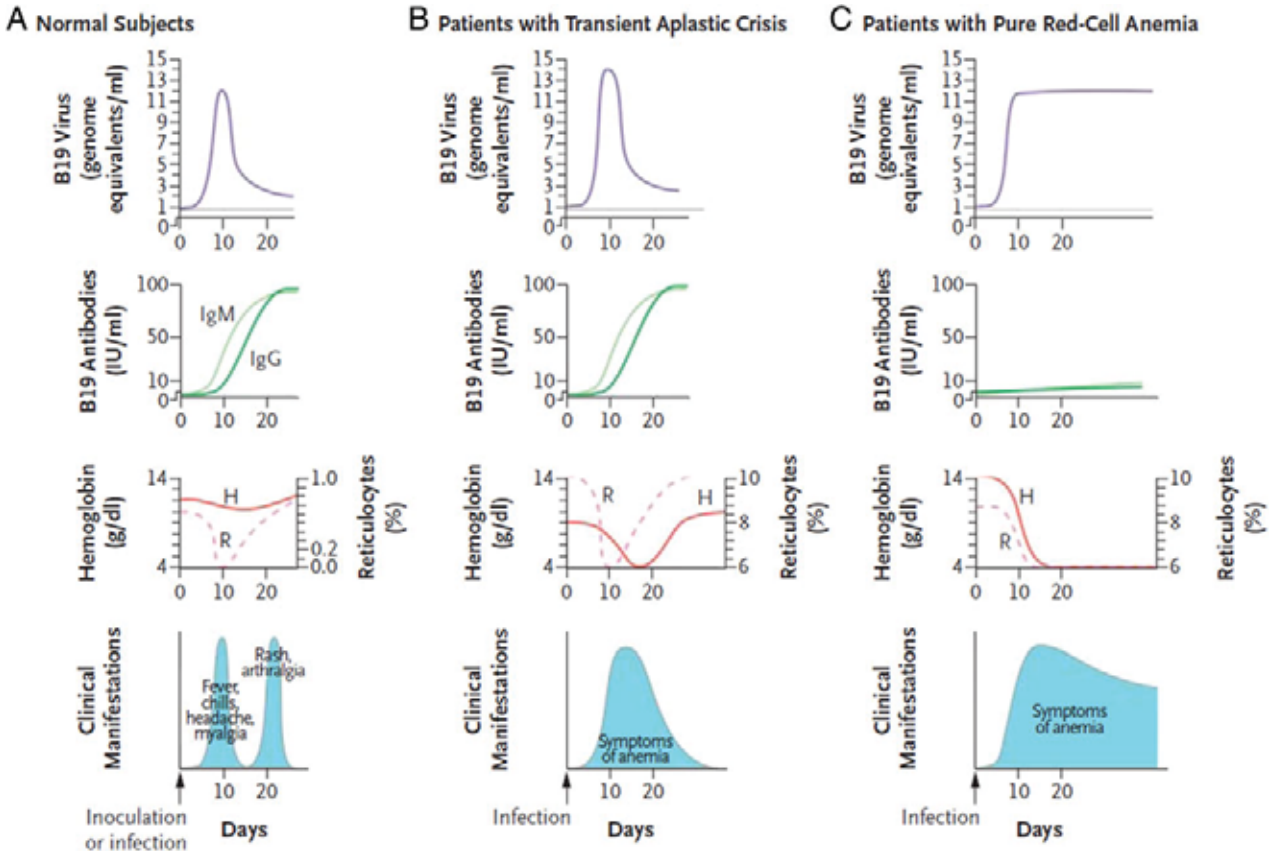

FIGURE 6 Pathophysiology of parvovirus B19 infection. Inoculation of normal subjects and natural infection resulted in fifth disease (Panel A; study reported by Anderson et al. [92]). A two-phase illness was produced under control conditions; most subjects noted only the typical rash, joint symptoms, or both, corresponding to the appearance of specific antiviral antibodies. Reticulocytopenia occurs during viremia, but hemoglobin levels do not decline below normal values. H denotes hemoglobin, and R reticulocytes. Transient aplastic crisis occurs in patients with underlying hemolysis or erythroid stress who are infected with parvovirus B19 (Panel B; study reported by Saarinen et al. [200]). Cessation of erythropoiesis causes severe anemia, because of the higher demand for red cells. Chronic pure red-cell aplasia is due to persistent infection (Panel C; study reported by Kurtzman et al. [203]). Anemia persists because of the failure of the humoral immune response to clear parvovirus B19. Reprinted from reference 3.

chronic lytic infection of red cell precursors and an associated chronic reticulocytopenic anemia (3, 159–161). Patients with chronic infection often respond to treatment with intravenous immune globulin (IVIG) with a prompt reticulocytosis and clearing of viremia. Chronic infection with anemia has also been rarely noted in patients with presumed normal immune systems (3, 159–161). Persistence of low levels of B19V in blood, bone marrow, synovial tissue, heart, and liver in adults from infection many years earlier complicates the task of linking B19V to disease, based on its detection in these tissues (144, 151, 162–165).

The fetus is susceptible to severe B19V anemia because it has a need for increased red cell production and an immature immune system that is not always able to control the infection (3). B19V-associated myocarditis may also contribute to fetal disease. Transplacentally transferred maternal antibodies provide some protection to the fetus during the later stages of pregnancy.

Although the major cell type for productive B19V infection is the erythroid progenitor, a number of other cells have the P-antigen receptor (45, 140) and may be positive for B19V DNA or antigens (144–146, 166, 167). *In vitro*, macrophage and endothelial infection have been facilitated by the presence of antibody, presumably through Fc receptor–mediated phagocytosis of virus-antibody complexes, or by the complement factor C1q receptor (168, 169). This may be the mechanism by which phagocytic cells in the bone marrow and synovial tissue and the endothelial cells in the heart may acquire B19 antigen and DNA. Further, B19V-infected bone marrow–derived circulating angiogenic cells may transport virus to diverse tissues where they could affect endothelial regeneration (e.g., resulting in cardiomyopathy) (170).

During respiratory tract infection, HBoV1 is often detected together with other respiratory pathogens. This, in large part, is due to nasopharyngeal persistence (or "shedding") of HBoV1 for many months, or even up to a year (171–173), which can complicate the interpretation of positive PCR test results (10, 174, 175). It is therefore more accurate to speak about co-*detections* rather than co-*infections*. Based on detection of single-nucleotide polymorphisms (SNP) or sequencing of HBoV1 DNA in follow-up saliva or nasopharyngeal secretions, a few studies have suggested the existence of re-infections or reactivations of latent HBoV1 (173, 176). However, contaminations from other infants, not leading to true infections in the immune children, were

not ruled out. If reactivations would occur, one would expect the HBoV1 detection rate to be high in elderly individuals, which is not the case (177). More information regarding the possibility of reactivations and reinfections and the role of immunological cross-protection is needed (93, 178). Whatever the reasons for prolonged persistence, disease descriptions should not be based on mere PCR detection but should be confirmed by other means (see Laboratory Diagnosis). The presence of HBoV1 DNA in blood seems to be more short-lived and may thus be a better marker for acute infection (93, 112). Similar to the presence of HBoV1 in the airways, the occurrence of HBoV2–4 in stool is prolonged, as is the case with many other enteric viruses (93, 179).

Immune Responses

IgM and IgG antibody responses (IgM and IgG) begin 10 to 14 days after infection (92) (Fig. 6) and correlate with reduction of the viral load in immunocompetent individuals. B19V IgG antibodies persist long-term and presumably confer protection from disease. The ability of IVIG to control and sometimes cure chronic infection in the immunodeficient patient clearly demonstrates the importance of antibodies in B19V immunity (160, 161). Various assays used to characterize the antibody response to B19V have identified differences in the temporal antibody response to epitopes on the capsid proteins: antibodies against linear epitopes on the VP2 protein are present during the acute phase but are lost in the late convalescent phase; VP1 linear and conformational epitopes are present during both the acute and the convalescent phases of infection (180, 181); neutralizing epitopes have been identified on both the VP1 unique and the VP1/VP2 shared regions of the structural proteins. An antibody response to the NS protein is detected in some patients (182–184). There is less information on the cellular immune response to B19V infection, although the evidence suggests that the low levels of B19V that can be detected in an individual following acute infection probably lead to persistent stimulation of the cellular response (185–191).

HBoV1 primary infections typically give rise to robust IgM and IgG responses with the latter persisting long-term at a relatively high level (112, 174, 192, 193). In contrast, HBoV2 and -3 IgM responses appear to be rare or short-lived in primary infections (93, 94). HBoV2 IgG responses also appear weaker and more prone to waning than those of HBoV1. This difference may reflect the frequency of systemic versus nonsystemic infections between these two virus types. In support of this notion, HBoV1 viremias coincide frequently with primary HBoV1 seroconversion, whereas corresponding HBoV2 viremias are infrequent.

Immune responses to HBoV1 infections are significantly weaker in individuals with preexisting HBoV2 antibodies and vice versa. A potential explanation is "original antigenic sin": i.e., the propensity of the immune system to become primed by an antigen and subsequently to disregard, partially or completely, the novel epitopes of related subsequent antigens (93, 95). Alternatively, or possibly as a comechanism, the data might be explained by cross-protection, with preexisting immunity against one bocavirus limiting the propagation of another.

Little is known about the immune responses to natural infections with AAV, BuV, and TuV. Antibodies can be found in both natural AAV infections and after AAV vector-mediated gene therapy. As in B19V infection, the immune response does not eradicate the virus from the body. Preexisting antibodies can, however, affect the safety and efficacy of AAV gene therapy by blocking transduction or directing the vector away from its target tissue (194, 195). Acute PARV4 infection does induce a specific antibody response, but due to the parenteral spread of the non-African genotypes of this virus, PARV4 IgG can mainly be observed among injecting drug users and hemophiliacs receiving clotting factors (99, 196). PARV4 infection may also elicit a strong T-cell response, which may be indicative of viral persistence (197).

CLINICAL MANIFESTATIONS OF B19V

Asymptomatic Infection

Asymptomatic infection or atypical illness is probably the most common manifestation of B19V infection. Among B19V-infected children in outbreaks (80, 86, 88), as many as 50% report no rash and 25% no symptoms.

Erythema Infectiosum

Erythema infectiosum or fifth disease, the most commonly recognized clinical manifestation of B19V infection (87, 198), is a mild illness manifested by a malar erythematous rash (slapped cheek) (Fig. 7) and reticulated or lace-like rash on the trunk and extremities (147). Erythema infectiosum is most commonly diagnosed in school-age children. The child is usually afebrile but may experience a mild systemic illness 1 to 4 days before onset of rash. Various nonrash symptoms have been noted in some patients, including headache, sore throat, coryza, pruritus, gastrointestinal symptoms, and arthralgias or arthritis. In most patients, symptoms resolve over the course of a few weeks, but in some cases they last months and, rarely, even years. A typical feature of erythema infectiosum is the recrudescence of rash after a variety of nonspecific stimuli, such as change in temperature, exposure to sunlight, or emotional stress. Patients with erythema infectiosum usually do not undergo laboratory evaluation but may have reticulocytopenia and clinically insignificant anemia, lymphopenia, neutropenia, and thrombocytopenia before and possibly at the onset of rash.

Transient Aplastic Crisis

Transient aplastic crisis (TAC) was the first illness associated with B19V infection (199). The lytic infection of the nucle-

FIGURE 7 Erythema infectiosum (fifth disease) associated with parvovirus B19.

ated red-cell precursors leads to cessation of hematopoiesis, which, in persons with a poorly compensated hematopoietic system, like sickle cell disease, may lead to severe, self-limited reticulocytopenic anemia, i.e., transient aplastic crisis. Patients present with symptoms of a severe anemia, i.e., pallor, weakness, and lethargy (200, 201), and sometimes also with nonspecific systemic symptoms and infrequently a rash. During the acute phase of the illness, patients have no reticulocytes and may have a drop in hemoglobin levels of 30% or more. The bone marrow has a hypoplastic or aplastic erythroid and normal myeloid series in the bone marrow. About 7 to 10 days after onset of illness, and coincident with development of an antibody response, reticulocytosis develops, and the hemoglobin begins to return to pre-infection levels.

Pure Red-Cell Aplasia

In patients who cannot mount an immune response, B19V infection causes a potentially life-threatening illness (148, 202–204). Some patients with deficient immune systems develop a chronic B19V infection and associated chronic, severe reticulocytopenic anemia or pure red-cell aplasia (159, 160).

Hydrops Fetalis and Fetal Death

Hydrops fetalis is a potential complication of B19V infection during pregnancy. The B19V parvovirus–infected fetus may not be able to control infection and develop a severe reticulocytopenic-anemia leading to high-output congestive heart failure, hydrops, and sometimes death (205, 206). B19V can also infect fetal myocardial cells, which may contribute to cardiac dysfunction and heart failure (3, 146). Although a few case reports have suggested that B19V might be teratogenic, most studies of children born of women infected with B19V during pregnancy have not demonstrated an increased risk of birth defects (74, 126, 132, 207–210). If B19 causes birth defects, it is likely an uncommon occurrence. B19V infection has, however, been detected in approximately 5% to 20% of autopsy cases of nonimmune hydrops fetalis (211, 212). Nevertheless, a hydropic fetus may also recover spontaneously or after intrauterine transfusion (see Treatment).

Approximately 30% to 50% of maternal B19V infections lead to fetal infection (130, 131). The risk of fetal death from an exposure can be defined by the following equation: rate of fetal death from an exposure = rate of susceptibility to B19V X expected rate of infection from the exposure X rate of fetal death with infection (213). The risk of fetal death with maternal infection during gestational weeks 9 to 20 is between 2% and 10% (74, 130–132, 211, 214–216), whereas the risk is negligible in >20 weeks of pregnancy (214). Maternal infection in the second half of pregnancy thereby presents much less risk to the fetus, possibly because transplacental transfer of maternal antibody and the maturity of the fetal immune response may occur and protect the fetus. Overall, the risk of fetal death in pregnancy can thus be estimated to be between 0.4 and 3.0% after exposure to B19V in the household and between 0.16 and 1.2% after exposures associated with working in a school or childcare setting with a B19V disease outbreak.

Arthralgias and Arthritis

Arthralgias and arthritis associated with B19V infection have been described in children and adults but most commonly in adult females (74, 130, 131, 211). This condition usually manifests as a symmetrical peripheral polyarthropathy commonly involving the hands, wrists, knees, and feet, although any joint can be affected. Joint symptoms may develop with or without other symptoms and before, during, or after the onset of rash and include tenderness and swelling. Symptoms usually resolve over the course of a few weeks but can persist for months and rarely for years. It is not a destructive arthritis. As with the rash of erythema infectiosum, joint symptoms can recur after a variety of stimuli.

The predisposition of B19V infection to cause joint disease has led to studies of B19V in patients with rheumatoid arthritis. While viral DNA is readily detected also in the joints of healthy individuals, there are suggestions of higher frequencies of DNA or B19V antibody in patients with rheumatoid arthritis, but these findings have been inconsistent (3, 151, 217). B19V is clearly not the cause of most forms of rheumatoid arthritis, although B19V-associated chronic arthritis may infrequently fulfill the classification criteria of rheumatoid arthritis, including induction of rheumatoid factor, but not anticitrullinated protein antibodies, and may similarly cause some cases of juvenile arthritis (218–221).

Myocarditis and Hepatitis

There have been a number of case reports of both myocarditis and of hepatitis associated with B19V infection. In many of the case reports, the diagnosis of B19V as the cause is made simply on the detection of the B19V DNA genome, and, given the known persistence of B19V DNA in tissues and at low levels in blood of healthy subjects (144, 167), this diagnosis may be erroneous. However, there are clearly some cases of acute myocarditis in children and adults (222, 223), and it is clear that B19V infection causes myocarditis in the fetus (38, 224, 225). However, the role of B19V infection in the etiology of chronic heart disease is much more questionable (226, 227).

Similarly, the role of parvovirus B19 in both acute and chronic hepatitis remains unclear. Transient elevation of liver transaminases is not uncommon in B19V infection, and frank hepatitis associated with acute B19V infection has been reported (228–231) but more infrequently. The role of B19V infection as a significant cause of chronic or fulminant hepatitis is more questionable (167, 232, 233).

Other Disease Associations

Other disease associations have been reported, but not established, for B19V infection. The list of possible B19V-associated conditions includes a wide range of cardiovascular, skin, endocrine, hematologic, neurologic, ocular, renal, respiratory, and rheumatic disorders (3, 234–236). Some of the purported associations are simply coincidental occurrences of a common infection and the disease. Others are probably instances in which B19V is one of several causes of the disease, and some represent instances in which false-positive laboratory results led to a spurious association. As with the persistence of B19V DNA in tissues mentioned above, false-positive results with B19V IgM antibody assays can be a substantial problem (144, 167, 237).

B19V infection may affect other lineages of the bone marrow, as well as the red cell precursors (98). There have been a number of reports of hemophagocytic syndrome associated with B19V infection in both healthy and immunosuppressed patients (238–240).

B19V infection has also been detected in patients with a wide range of rashes, including morbilliform, vesicular, and confluent ones, in addition to the slapped cheek and

reticular, lace-like rash on the trunk and extremities characteristic of erythema infectiosum. Other dermatologic illnesses reported with B19V infection include papular purpuric "gloves and socks" syndrome and papular acrodermatitis of childhood, Gianotti-Crosti syndrome, and livedo reticularis (241–243). Case reports of vascular purpura were first vasculitis-like findings associated with B19V infection (244). However, reports of B19V infection being associated with other vasculitidies, including Henoch-Schönlein purpura, Wegener's granulomatosis, polyarteritis nodosa, and Kawasaki disease, have not been substantiated (3).

B19V infection has been associated uncommonly with various neurologic abnormalities, including peripheral neuropathies, meningitis, and encephalopathy (245). B19V DNA has been detected in cerebrospinal fluid from several patients with encephalitis or meningitis during acute B19V infection (246), and B19V antigens and DNA have been detected in fetal brain tissue (247). These findings suggest that B19V infection may occasionally lead to neurologic disease.

Clinical Illness Associated with the Human Bocaviruses

HBoV1 causes subclinical to life-threatening acute respiratory infections and has been implicated also in encephalitis (11, 248–250). HBoV1 is detected typically in 1 to 20% of respiratory specimens from children with acute respiratory illnesses (108, 109, 111, 174, 251, 252), whereas it seems to be much less common in adults and the elderly (10, 177, 253). The illness in HBoV1-positive patients is indistinguishable from that caused by other respiratory viruses and includes upper respiratory tract illness and a range of lower respiratory tract manifestations, such as pneumonia, bronchitis, bronchiolitis, and exacerbation of asthma (109, 110, 251, 254, 255). Typical symptoms often emphasized though are cough, wheezing, fever, acute otitis media, and diarrhea. The role the virus plays in the illness, however, has been confounded by detection of co-infecting viruses in as many as 90% of specimens, and presence of HBoV1 has also been detected in asymptomatic children. This, however, is due to the long-term persistence of this virus in the airways, which necessitates other diagnostic means than mere PCR in airway samples (10, 109, 171–175, 251, 256, 257) (see Laboratory Diagnosis). Particular pediatric symptoms that have been statistically associated with HBoV1 or confirmed by serodiagnosis are wheezing and pneumonia (109, 174, 250, 251, 258, 259). Positive correlations have been observed also between respiratory illnesses and high HBoV1 viral loads or HBoV1 sole infections (10, 174, 251, 260, 261). In a pediatric intensive care unit, seven children with severe acute respiratory tract illness (respiratory failure, bronchiolitis/asthma) were identified who had HBoV1 as the sole pathogen, as evidenced by next generation sequencing (NGS) (261). Longitudinal weekly saliva sampling and health data collection during the first two years of life have further provided evidence that HBoV1 infection was associated with new onset of upper respiratory symptoms (i.e., cough, rhinorrhea), as well as with health care visits (173). Despite the diagnostic challenges, accumulating evidence thus strongly suggests that HBoV is an important respiratory pathogen in children. Conversely, HBoV1 does not seem to be a major cause of gastroenteritis in constitutionally healthy children (262–264), even though one case report describes a life-threatening hypovolemic shock due to diarrhea associated with disseminated HBoV infection in an immunocompromised child (265).

HBoV1 has also occasionally been detected in adults with respiratory tract illnesses. Two adults with idiopathic pulmonary fibrosis (IPF) were shown to harbor HBoV1 in bronchoalveolar lavage (BAL), in one, as the sole pathogen, and in the other, codetected with cytomegalovirus (CMV) (266). In the latter patient, both viruses were also present in various postmortem tissues. Furthermore, an immunocompromised adult with rapidly progressing fatal pneumonia had HBoV1 as the sole pathogen in blood ($1.5x10^4$ copies/ml), and, as codetection with EBV, rhinovirus, and *Candida albicans*, very high HBoV1 loads in tracheal secretions ($4.8x10^{10}$ copies/ml) and sputum ($4.3x10^9$ copies/ml) (267), indicative of an acute infection at the time of death. Among both immunocompromised and immunocompetent adults, HBoV1-positive cases seem to be symptomatic (having mainly pneumonia or acute respiratory insufficiency), whereas nonsymptomatic controls tend to be HBoV1 negative (268, 269). This would suggest that in adults, HBoV1 does not occur as a bystander; instead it seems to be a real, but infrequent, cause of respiratory symptoms. Moreover, in HBoV1 infection of human airway epithelium, air-liquid interface cultures show hallmarks of respiratory tract injury, such as loss of cilia, epithelium thinning, and hypertrophy (69).

The enteric HBoV2 has been associated with gastroenteritis in one report but not in others (5). HBoV2, like HBoV1, occurs in 1 to 20% of stool specimens, and, occasionally, even in blood of children both with and without acute gastroenteritis (111, 262–264, 270, 271). HBoV2, as the single finding in various tissues, blood, or CSF, has been implicated in a fatal case of myocarditis and of encephalitis (248, 272); HBoV3–4 lack any known clinical role, although HBoV3 was found as a single agent in the CSF of a child with encephalitis (11).

Clinical Illness Associated with Other Parvovirus Infections

AAVs, PARV4, BuV, and TuV have not yet been linked to human disease, although BuV has infrequently been detected in stools of patients with diarrhea (102, 273). In one recent study, the number of diarrheal stools per day in BuV-positive children was also significantly greater than in BuV-negative children, including norovirus- or rotavirus-positive children (102), suggesting a causative association. Only a few acute PARV4 infections have been recorded, mainly in injecting drug users or hemophiliacs, with no discernable PARV4-specific symptoms (99, 196). The apathogenicity of AAV is a valuable reason for its application as a tool for gene therapy (12).

LABORATORY DIAGNOSIS

Serologic, antigen, and nucleic acid detection assays have been used for diagnosis of B19V infection. Acute infection in the healthy patient is most often demonstrated by detection of B19V IgM and past infection by detection of B19V IgG antibodies. Antibody assays are not reliable for diagnosing infection in immunocompromised patients, in whom detection of B19V DNA by PCR is required.

Detection of AAV, HBoV, PARV4, and BuV has been primarily accomplished with PCR-based assays. However, mere PCR-positivity in the airways, stool, or tissues is not a diagnostic marker of a primary infection. The diagnostic criteria for parvovirus primary infections in general are IgG seroconversion, IgM positivity, low-avidity IgG, and

detection of DNA in blood plasma (99, 174, 237, 274). Past infection has been determined using a variety of serologic assays for IgG antibodies (181, 237, 274, 275).

Virus Isolation

Although erythroid precursor cells derived from human bone marrow, peripheral blood, and several continuous cell lines with erythroid precursor-like features support B19V infection, they do so inefficiently, and isolation has not been used to detect infection (55, 64). Isolation studies have been used to evaluate inactivation methods for blood or plasma products (72). HBoV1 can be cultured in a differential air-liquid interphase epithelial cell culture, which cannot be applied in a routine diagnostic laboratory (68, 69). There are no *in vitro* culture systems yet for PARV4, BuV, or TuV.

Antigen Detection

Enzyme immunoassays have been developed for B19 parvovirus antigens, but they lack sufficient sensitivity to reliably diagnose acute infection, although they can be used for screening blood and blood products (276–278). Immunohistochemical techniques have proven useful for detecting B19V antigens in various tissues and cells, most commonly in fetal tissues and bone marrow specimens (139).

For detection of HBoV1 in nasopharyngeal samples, a less sensitive test, such as an antigen test, may be a better alternative than PCR. HBoV1 has recently been included in a multiplex point-of-care antigen test for respiratory tract infections, which is based on separation-free two-photon excitation fluorometry (279–281).

Nucleic Acid Detection

Nucleic acid detection is an important part of B19V diagnostics for both detecting infection and screening blood products. Probe hybridization assays used in the past have been replaced by PCR assays, especially real-time qPCR assays that have the advantage of both speed and quantitation. A multitude of in-house and a few commercial PCR assays have been developed using a variety of primers and methods (282, 283). The sensitivity and specificity of PCR varies among assays and laboratories (284). It is critical that assays should be designed to detect all three B19V genotypes and be calibrated against the WHO B19V nucleic acid standard if quantitative results are desired (285). Due to the sensitivity of PCR, B19V DNA can remain detectable for months or even years at low levels, even following complete recovery, and thus quantitative PCR is required to distinguish recent infection (viral loads $>10^6$ IU/ml) from past infections (237).

In situ hybridization studies have been successfully used to detect which cells and tissues harbor B19V DNA (139). Additional detection of viral mRNA can be useful for confirming the presence of replicating virus and active infection. Often this is achieved by amplification across a spliced junction to distinguish spliced RNA from viral DNA (286).

Most recent studies of HBoV1–4, AAV, and PARV4 have been based on a variety of PCR assays to detect the viral DNA (13, 23, 196, 254, 270, 287). Due to the prolonged nasopharyngeal persistence of HBoV1 for as long as a year, detecting the presence of HBoV1 by conventional PCR is not diagnostic (10, 172–174). Fortunately, other methods have been developed that more accurately indicate acute primary HBoV1 infection, such as serology (to show IgM positivity, IgG seroconversion, and low IgG avidity), RT-PCR in airway samples (to demonstrate transcription activity of HBoV1, suggesting replication), qPCR (to separate high from waning lower viral loads in the nasopharynx), PCR in serum (revealing the typical brief viremic phase of an acute HBoV1 infection), and also antigen detection (174, 192, 193, 258, 281).

Antibody Assays

B19V IgM antibody assays are the key to diagnosing most infections in immunocompetent individuals. A mu-capture antibody format as an enzyme immunoassay is the preferred format, with conformational epitopes as antigen (42, 181). Expressed proteins that do not form empty capsids may not include all the epitopes needed to detect a response in all patients, although they can be useful as antigens for timing when an infection occurred (181).

IgM antibody develops within 10 to 12 days after infection and is present in more than 90% of patients with erythema infectiosum at onset of rash and in about 80% of patients with TAC at the time of presentation (113, 276, 277, 288). IgM positivity increases to more than 90% in patients with transient aplastic crisis by 3 to 7 days after presentation. The IgM response begins to wane during the second month after infection. Indirect IgM antibody assays have been used but tend to be both less sensitive and less specific than the IgM capture format (289, 290). The capture assay method eliminates competition from IgG antibodies that can lead to false negative results and decreases the risk of rheumatoid factor or nonspecific sticking of IgM antibodies that can lead to false positive results.

Other serologic indicators of acute or active B19V infection include low-IgG avidity (275, 291) and presence of antibodies against linear VP2 epitopes, i.e., epitope-type specificity (180, 181, 292). IgG antibodies appear at the same time as IgM antibodies and persist long-term. Unlike IgM antibody assays, the IgG indirect format should be as good as, or better than, the capture format.

As with B19V, HBoV antibody assays have been based mostly on empty virus-like particles (VLP) expressed in insect cells infected with recombinant baculoviruses (174, 192, 193, 259, 293–298). However, detection of HBoV1–4 immune responses is complicated by the structural similarity of these viruses, manifesting as serological cross-reactivity and "original antigenic sin" (93–95). This currently necessitates the use of antibody absorption assays to measure HBoV type-specific antibodies. Serological diagnosis of primary HBoV1 infections in individuals without preceding HBoV2–4 antibodies is considered highly specific and sensitive. Measurement of HBoV1 IgG-avidity is also helpful for confirming a primary infection (274). However, HBoV1 infections in individuals with preexisting HBoV2 IgG may be difficult to detect serologically, because HBoV1 antibody responses in these children are typically weak or even nondetectable. Primary HBoV2 antibody responses appear to be similarly affected by preexisting HBoV1 immunity. The sensitivity of serodiagnosis of HBoV2–4 primary infections is not well known because comparative data on the presence of HBoV2–4 DNA in stool and HBoV2–4 antibodies in blood are lacking.

A variety of serologic assays have been used to detect AAVs in a serotype-specific fashion (96, 194, 299). Serologic assays for PARV4 using recombinant VP2 capsids have been used for diagnosis of acute infection and for seroprevalance studies (97–99, 196).

PREVENTION

Since B19V and HBoVs are probably most often transmitted via respiratory secretions during close contact, possibly by droplet or aerosol transmission, attention to good hygienic practices, like hand washing and not sharing food, may be effective in reducing the spread of both of these viruses. Although the effectiveness of such measures has not been studied, these public health interventions are straightforward and might reduce spread during outbreaks. For HBoV infections, as with any respiratory infection, distancing from persons with symptoms is also appropriate.

However, for B19V, respiratory symptoms are not a primary feature of infection, and, although nosocomial transmission has been reported after exposure to patients with TAC and chronic anemia due to B19V, once individuals have developed the rash the patient no longer has high viral loads and low levels or no virus in respiratory secretions (3, 113). Patients with TAC are probably a risk for transmitting B19V as long as a week after onset of illness and should be placed on droplet-isolation precautions for 7 days. The risk for transmission from immunodeficient patients is less well understood but may be prolonged, and immunodeficient patients with chronic infection should be placed on droplet precautions for the duration of their hospitalization (125).

Persons should be informed of potential exposures to B19V, and efforts to decrease the risk of exposures (e.g., avoiding the workplace or school environment) should be made on an individual basis after consultation with family members, health care providers, public health officials, and employers or school officials (213). Seronegative pregnant women should avoid contact with B19V-infected children (e.g., daycare centers experiencing outbreaks), at least during the first half of pregnancy (129, 214).

Although currently there are no vaccines available for B19V or any of the human parvoviruses, due to the ease of production and the neutralization efficiency of VLPs, prospects for a vaccine against B19V are favorable. A vaccine made from empty capsids produced in insect cells has been developed and evaluated (300); however, Phase 1 trials were halted due to unexpected cutaneous reactions in three patients (301). A second vaccine made in yeast is under development (302).

TREATMENT

No viral-specific chemotherapy is available for B19V infection; however, most B19V illnesses, including erythema infectiosum, are mild and require no treatment. B19V-associated arthralgias or arthritis may benefit from nonsteroidal, anti-inflammatory medications. Patients with TAC often require hospitalization and transfusion therapy until the immune response controls the infection 1 to 10 days after presentation. In one study of 62 patients with sickle cell disease who developed TAC, 54 (87%) required blood transfusions (303). The death of one patient before transfusion therapy highlights the importance of prompt evaluation and treatment of patients with transient aplastic crisis (201).

Serum from blood donors, who have recovered from B19V infection, generally have high titers (1:200–1:2,500) of neutralizing antibody (286, 304), and immunocompromised patients with chronic infection and associated anemia should be treated with IVIG. Commercial IVIG, at a dosage of 400 mg/kg for 5 or 10 days or at a dosage of 1 g/kg for 3 days, has been used successfully (161, 203). Patients often respond to IVIG with a brisk reticulocytosis and resolution of B19V-associated anemia and a drop of the viral load to <10 copies/ml. In some patients, the symptoms may resolve completely (although low-level B19V DNA can still be detected), whereas in others, high-level viremia and anemia may recur, resulting in the need for additional treatments with IVIG (305). Patients can be monitored with hemoglobin and reticulocyte counts, and if they develop a reticulocytopenic anemia and recurrent high B19 viral load, then retreatment with IVIG is indicated. IVIG treatment has not been shown to be effective for treatment of nonanemia manifestations of chronic infection (306).

Management and treatment of the fetus of a B19V-infected pregnant woman are problematic. Most studies have advocated monitoring the fetus with ultrasound to look for evidence of hydrops and treating hydropic fetuses with intrauterine blood transfusion. However, since the fetus can survive and be normal without treatment and intrauterine blood transfusion can cause fetal death, this approach to managing the hydropic fetus needs to be considered carefully. Intrauterine blood transfusion may be beneficial to an affected fetus, but the efficacy of this treatment is variable (205, 307–309). In one study, 9 of 12 fetuses treated with intrauterine blood transfusions survived and were normal at delivery compared to 13 of 26 untreated ones (307); analysis indicated a significant difference after adjusting for severity of ultrasound findings and gestational age. Although most B19V-infected transfused fetuses that survive are normal, one study of 24 transfused hydropic fetuses reported that some suffered long-term complications (208), including delayed psychomotor development in 5 of the 16 survivors (severe in 2). The available data suggest that intrauterine blood transfusions may be helpful and should be considered in the management of some fetuses with B19V-associated disease. Unfortunately, the data do not clearly indicate when the benefits of this procedure outweigh the risks.

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Anelloviridae

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31

The family *Anelloviridae* includes the human torque teno virus (TTV) and related small nonenveloped viruses with circular single-stranded DNA genomes. Viruses of this family frequently or ubiquitously infect humans and a range of other mammalian species. Infections are characterized by their lifelong persistence and great genetic variability. Despite the original claimed association between anellovirus infection and hepatitis in humans when first discovered in 1997, no evidence convincingly links infections with anelloviruses to clinical disease.

VIROLOGY

Discovery

TTV was identified in the plasma of an individual who developed non-A to E hepatitis after blood transfusion, using representational different analysis (RDA). RDA provided the method for the specific amplification of nucleic acid sequences present in the plasma of the individual (initials "TT") during the period of his acute hepatitis but absent before transfusion (1). The cloning of the newly christened "TT" virus provided nucleotide sequences that allowed the development of methods for its detection by PCR. The original PCR method used primers from the "N22" region (a part of the gene encoding the TTV structural protein; see Composition) and were used to investigate other cases of posttransfusion hepatitis and other liver diseases of unexplained etiology (see Pathogenesis).

The cloning of the N22 region allowed the sequence of the rest of the TTV genome to be determined and its genome organization to be characterized (2, 3). The genome is comprised of single-stranded DNA with coding sequences on the antigenomic strand. The length of the genome of the originally described (prototype) isolate of TTV (TA278) was 3,853 bases in length, forming a covalently closed circle (2). This genomic configuration is most similar to that of the animal virus, the chicken anemia virus (CAV), with some similarities in its arrangement of coding sequences.

In the years following the discovery of TTV, further highly divergent groups of TTV-like viruses have been found in humans, in nonhuman primates, and in a variety of other mammalian species. Independently, a virus also having a claimed association with posttransfusion and other forms of hepatitis was discovered by RDA. Initially described as SEN virus (SENV) after the initials of the infected patient (4), it became apparent that it represented a different genotype of TTV (see Genetic Variability). More divergent human viruses include the TTV-like minivirus (TTMV) and the TTV-like midivirus (TTMDV). TTMV was accidentally discovered by PCR of human plasma samples using TTV-specific primers that matched homologous sequences in TTMV but generated a noticeably shorter amplicon than expected for TTV (5). Its overall genome length was additionally much shorter than TTV (approximately 2.8 kilobases). Further TTV-like variants, initially described as small anellovirus types 1 and 2 (SAV-1 and SAV-2) because of their shorter genome lengths than TTV and TTMV, were cloned from a plasma sample of an individual at high risk for human immunodeficiency virus (HIV) infection (6). Subsequent findings indicated that the originally published SAV-1 and SAV-2 sequences were incomplete (7) and, given their finally determined sizes of 3.2 kilobases, these were renamed the TTMDVs.

A large number of anellovirus species have subsequently been reported in wild and domesticated animals, including pigs, wild boar, camels, cats, dogs, pine martens, badgers, sea lions, and a number of nonhuman primates (8–23). Many of the initial discoveries were made using PCR with highly conserved primers from the untranslated region, but novel species are increasingly being identified using sequence independent methods involving rolling circle amplification followed by random cloning or high throughput sequencing.

Classification

TTV and related viruses are currently classified in the recently created family *Anelloviridae* containing 11 officially recognized genera and 66 species (24, 25). The human viruses TTV, TTMV, and TTMDV (along with phylogenetically interspersed viruses of nonhuman primate origin) comprise the *Alphatorquevirus*, the *Betatorquevirus*, and the *Gammatorquevirus* genera, respectively. The remaining genera (*Deltatorquevirus*, *Epsilontorquevirus*, *Zetatorquevirus*, *Etatorquevirus*, *Thetatorquevirus*, *Lambdatorquevirus*, *Iotatorquevirus*, and *Kappatorquevirus*) contain viruses identified in tupaias, tamarins, douroucoulis, cats, dogs, sea lions, and two viral genera in pigs, respectively (Fig. 1). The highly diverse range of genome sizes of anelloviruses (from approximately 2

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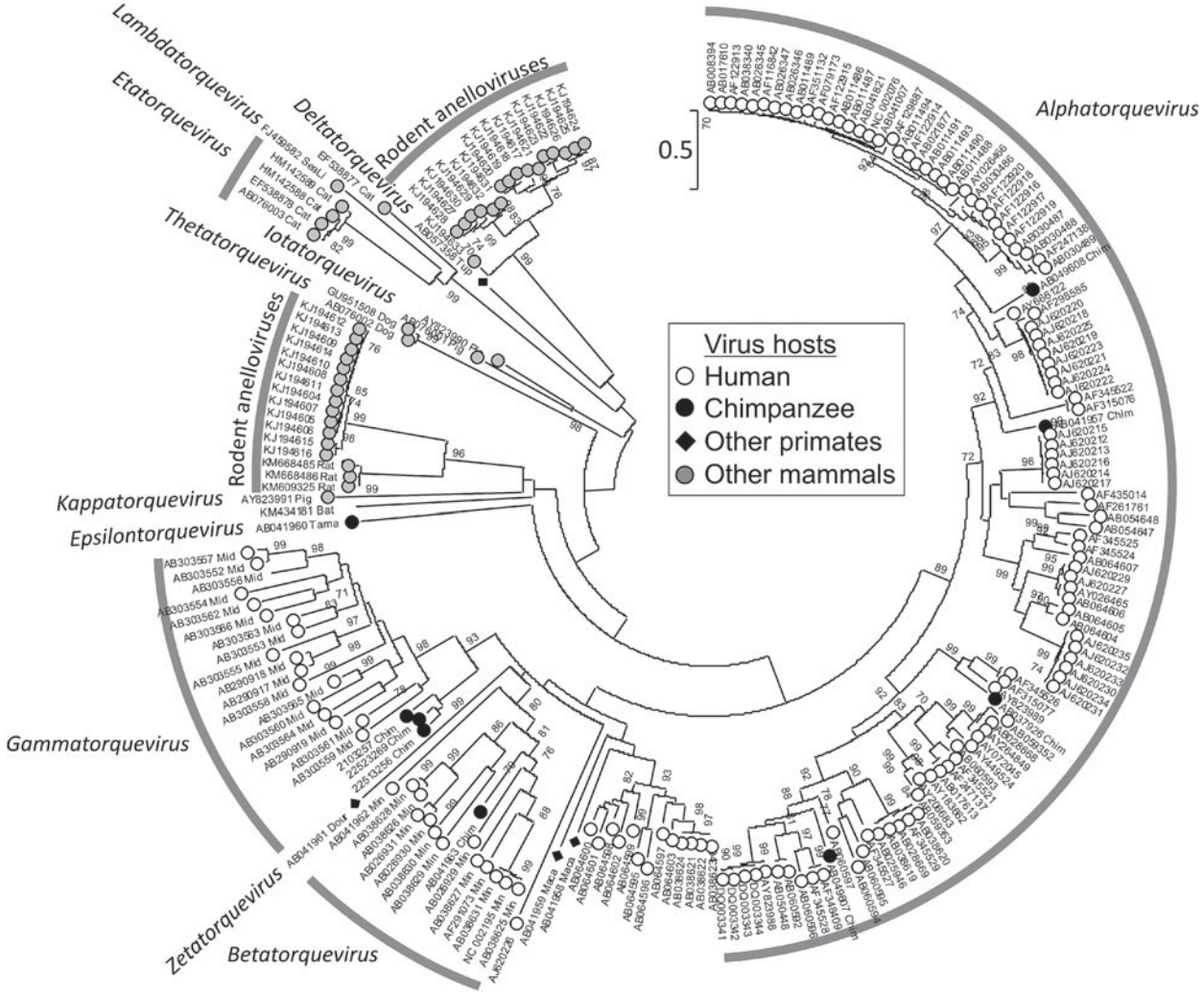

FIGURE 1 Diversity of anelloviruses infecting humans, nonhuman primates, and other mammalian species. Sequence divergence of different anellovirus genera in the ORF1 protein-coding sequence. An alignment of ORF1-coding sequences between positions 1–862 (numbered from the start of the reading frame of the TA278 [NC_002076] reference sequence) was created using MUSCLE, and a maximum likelihood phylogenetic tree was constructed using the optimal model (LG distances + F + G). The tree was bootstrap resampled to indicate robustness of groupings; values of greater than or equal to 70% are shown. Genera have been labelled according to the current International Committee on Taxonomy of Viruses (ICTV) classification, although several sequences currently classified as members of *Alphatorquevirus* do not cluster phylogenetically with other members of this genus (indicated by the dotted line). Sequences from nonhuman primates, such as the chimpanzee and monkey species (•, ◆), were frequently interspersed within the human-derived variants, while those from nonprimate mammalian species (e.g., pig, cat, dog, and rodent) formed separate groupings.

to approximately 4 kilobases) renders reliable taxonomic classification based on full-length sequences impossible, and classification is currently based on the analysis of the full-length ORF1 at the nucleotide level. Given the relatively high sequence variability in these viruses, the divergence cut-off value for genera is greater than 56%, and for species it is greater than 35%. With the rapid discovery of novel anellovirus or anellovirus-like sequences in a wide range of species, the number of officially recognized genera is likely to increase substantially in the future.

Genetic Variability

Anelloviruses are characterized by extreme genetic diversity (Fig. 1). Not only does this create considerable problems for the classification of human TTV variants, but it also hampers the development of methods for anellovirus screening, genetic characterization, and genotype identification. The situation has been made more complex by the discovery of TTMV and TTMDV that contain their own sets of genotypes and genetic groups and subsequently by the existence of a wide range of homologues of TTV and TTMV in nonhuman primates, as well as even more divergent viruses in other mammalian species (Fig. 1) (8, 11–23, 26, 27). Although the phylogenetic tree in Fig. 1 is based on the majority of publicly available complete anellovirus genome sequences, it likely only depicts a small subset of these viruses present in nature. The most conserved region of genome between all anelloviruses is in the untranslated region (UTR) (Fig. 2). PCR using primers from this region can amplify each of the five main genetic groups of human TTV, as well as TTMV, TTMDV variants, primate TTV-like viruses, and the majority of other related mammalian viruses.

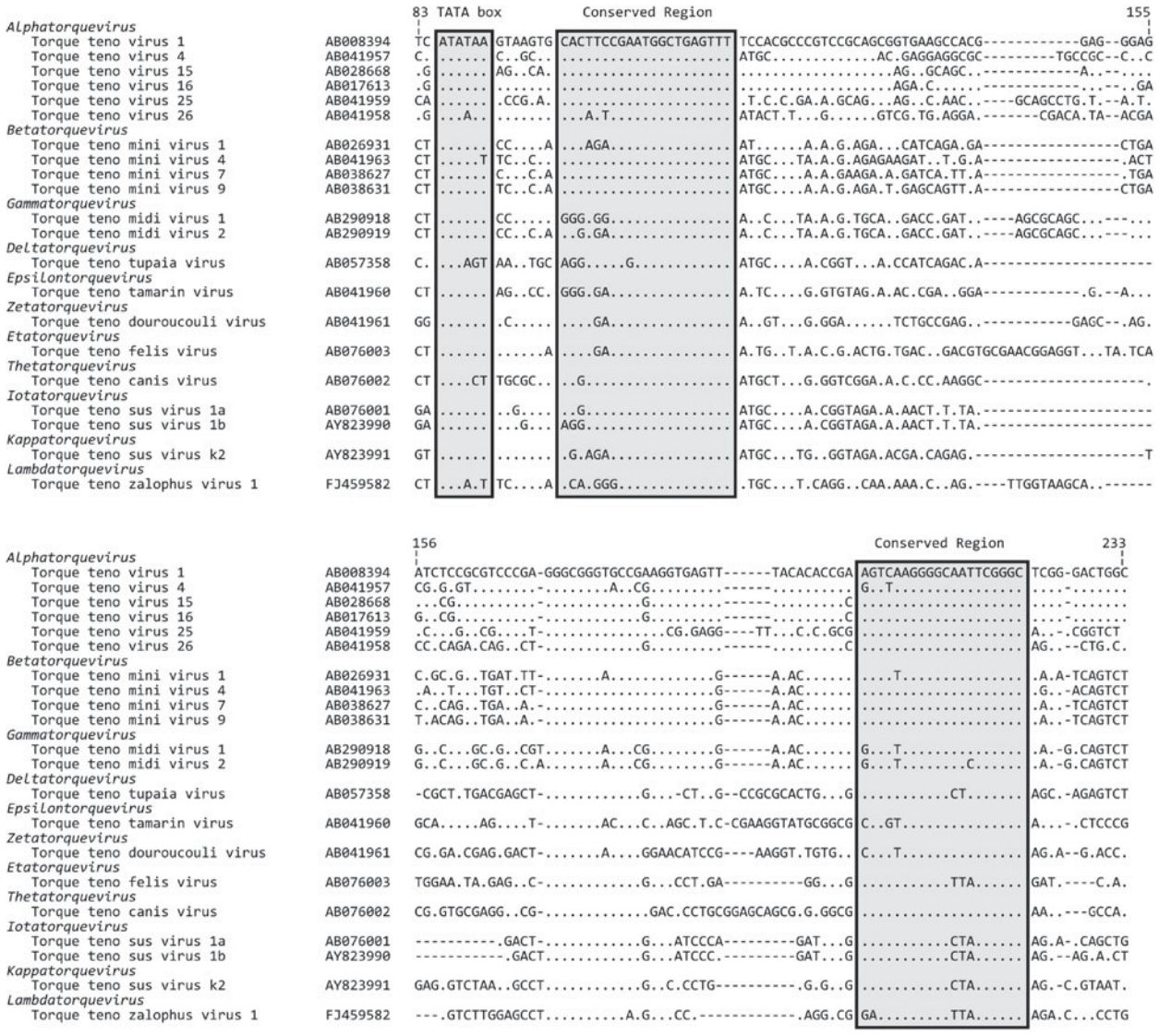

FIGURE 2 Alignment of sequences from the untranslated region of anelloviruses infecting humans and other species. Alignment of nucleotide sequences from the UTR of classified anelloviruses. Highly conserved regions are indicated in gray-shaded boxes, including the putative TATA box at position 85 in the prototype TTV sequence, TA278 (NC_002076). Symbols: ".": sequence identity with prototype TTV sequence; "-": gap introduced to preserve alignment of homologous nucleotide sites.

Among nonhuman primates, TTV variants are species specific; for example, chimpanzees harbor a range of TTV genotypes distinct from those infecting humans, although they generally fall within the genetic groups of human viruses (Fig. 1). TTV sequences from primates more distantly related to humans such as macaques and the New World tamarins and owl monkeys are increasingly divergent from TTV variants infecting humans (Fig. 1) (22). TTMV infection has been detected in chimpanzees, with some reports showing genotypes interspersed with human genotypes (13, 16) and another finding phylogenetically distinct sequences (28). A complication of primate studies is the cross-species transmission of TTV genotypes; for example, it is known that human TTV variants can infect chimpanzees and macaques (29, 30). Cross-species transmission may occur in captivity or through the administration of human plasma-derived blood products containing infectious anelloviruses, which frequently occurs in experimental animals. In the future, samples should ideally be collected from animals in the wild to investigate further their species specificity.

Composition

Anelloviruses are nonenveloped small viruses with a diameter measured by electron microscopy of 30 to 32 nanometers. The density of TTV in plasma on sucrose gradients ranged from 1.31 to 1.34 grams per cubic centimeter, although it was slightly higher for TTV virions excreted in feces (1.34 to 1.35 grams per cubic centimeter) (31). TTV purified from the plasma of infected individuals was found to be frequently immune complexed with IgG (31). The virion is likely composed of copies of only a single viral protein. TTV and related viruses are stable in the environment and can be detected following nanofiltration of blood products spiked with positive sera (32). Infectious TTV can be recovered from human feces (33), and frequent fecal shedding

even from healthy individuals has been reported (34). Based on the ubiquity of the virus in waste and its stability, the detection of anellovirus DNA has been proposed as a more appropriate biomarker for viral pathogen contamination of water sources (35, 36), hospital surfaces, and even air (37, 38) than currently used indicator systems based on the detection of coliform bacteria.

The genomes of anelloviruses contain several open reading frames (ORFs) that code for several putative viral proteins from either spliced or unspliced mRNA transcripts (Fig. 3). In the case of TTV, the antigenomic sequence ranges from 3,750 to 3,900 bases in length, in which a total of four gene sequences can be consistently identified among the different genetic groups. The largest open reading frame (ORF1) potentially encodes a protein of 770 amino acids in the TTV prototype strain TA278, while homologous proteins in related TTMV and TTMDV human viruses are shorter at 673 and 663 in prototype strains (Fig. 3). Based on an analogy with CAV, the ORF1 product is likely to form the replication-associated protein as well as the nucleocapsid (39), with perhaps the amino terminus being responsible for binding, encapsidation, and nuclear targeting of genomic DNA. ORF2 sequences are also observed in all anelloviruses, and their translation is predicated to occur through the use of an initiating methionine upstream of ORF1.

The presence and nomenclature of other predicted ORFs in other anelloviruses is less conserved, and their translation requires the splicing of viral mRNAs. In the case of TTV, three main species of mRNA of sizes 2.8, 1.2, and 1 kilobases have been detected after *in vitro* transfection of TTV DNA (40) and in bone marrow and liver tissues *in vivo* (41). All mRNAs share common 5' and 3' ends (Fig. 3); for the large transcript, splicing removes an intron between positions 185 and 277, leaving the entire coding sequences of ORF1 and ORF2. For the shorter 1.3-kilobase mRNA, splicing removes the sequence between positions 771 and 2374 to join the coding sequence ORF2 with that of ORF4. The 1-kilobase transcript is generated by the removal of an intron from 771 to 2564 or 2567 to join ORF2 to ORF5. Each transcript can be translated from initiating methionines at positions 354 or

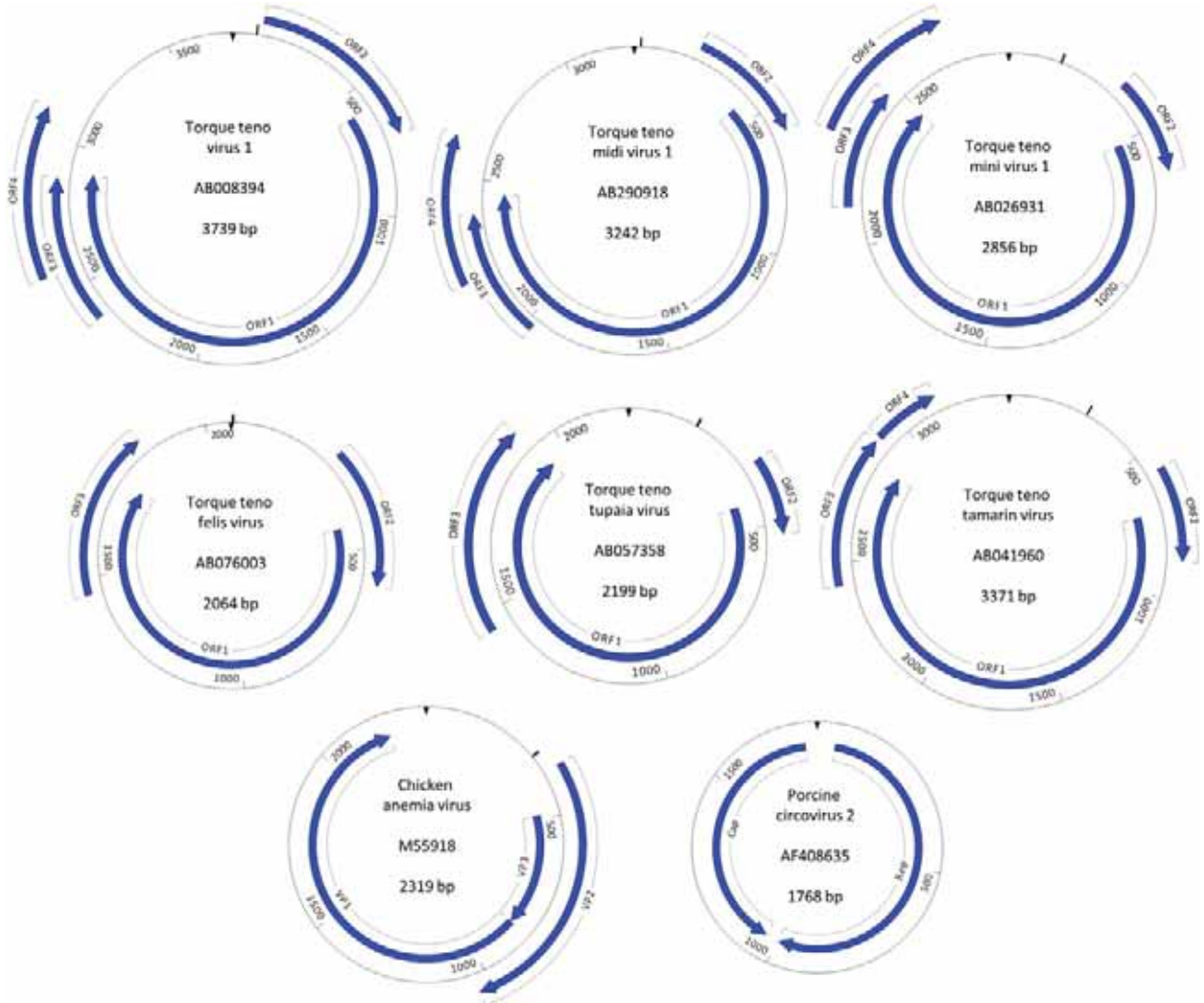

FIGURE 3 Genome organization of anelloviruses. Genome organization of representative anelloviruses showing arrangement of genes on the antigenomic strand. Closed arrows represent the principal open reading frames in each virus. The genome organizations of two other small circular ssDNA viruses (chicken anemia virus and porcine circovirus 2) are shown for comparison.

581; thus, a total of six distinct proteins can be expressed during replication (42). TTMV and TTMDV show the same arrangement of ORFs as TTV, and it is therefore likely that nonstructural proteins comparable to those from ORF2/ORF4 and ORF2/ORF5 would be produced, as well as the structural protein from ORF1.

The UTR of the TTV genome contains conserved promoter sites, a TATA box, and cap sites consistent with the transcription of mRNAs from position 98 and termination around the polyadenylation signals at position 3073. The region contains a number of transcription promoters and regulatory elements responsible for transcriptional control (43). Although the nucleotide sequences of the UTRs of other anelloviruses are distinct, there are some restricted regions of sequence identity or close sequence similarity interspersed with more variable regions (Fig. 2). These regions of clear sequence homology are conserved between human TTV and the more divergent human and animal anelloviruses and can even be found in the otherwise dissimilar UTR of CAV. Apart from the TATA box, the functional constraints that have retained this sequence similarity are uncertain.

Replication

Anelloviruses are likely to replicate in the nucleus of the infected cell, using host polymerases. The replication of small DNA viruses, such as the parvoviruses, is cell cycle dependent, and their replication is confined to rapidly dividing cells, such as those found in the bone marrow, the gut, and fetal tissue. It is unknown whether the replication of anelloviruses is restricted in a similar way or whether anelloviruses encode proteins with transforming activity, perhaps corresponding to the T antigens of the polyomaviruses that drive cells into division.

TTV can be cultured in the laboratory by the inoculation of plasma-derived virus onto Chang liver cells (44) or stimulated peripheral blood mononuclear cells (PBMCs) (45, 46). However, in contrast to the relative ease of virus culture of CAV and circoviruses, TTV replication *in vitro* occurs at low levels, and serial passage of virus is rarely achieved. Cloned TTV genomic DNA can also be replicated *in vitro* in cells engineered to express the SV40 large T antigen, but the ability of different isolates to propagate infection is highly variable (47). Replication in this system was also found to result in the production of replication-competent subviral genomes akin to those detected in sera from pregnant mothers whose children later developed childhood leukemia (48).

Anellovirus infections *in vivo* are characterized by persistent lifelong viremia, with circulating TTV levels typically ranging from 10^4 to 10^6 DNA copies per milliliter detected in humans and nonhuman primates (49, 50). Levels of viremia reflect the balance between virus production and clearance from the circulation by the immune-complex formation and the destruction of virus-infected cells; it has been estimated that a daily production rate greater than 10^{10} virions per day is required to maintain observed levels of viremia (51).

The sites of this *in vivo* replication remain a debated topic. Initial investigations concentrated on the liver, given the originally described association of TTV infection with posttransfusion hepatitis, and *in situ* hybridization or quantitative PCR have provided evidence for replication of TTV in the liver (52–54), a conclusion corroborated by the detection of double-stranded replicative intermediates in this tissue (55) and high levels of TTV excreted in bile (33, 56, 57). The latter may be the main source of TTV in the gastrointestinal (GI) tract and its excretion in feces. TTV DNA has been detected in virtually all other tissues tested (58–60). Double-stranded, presumed replicative intermediates of DNA have been detected not only in liver but also in stimulated PBMCs, lung, lymph node, thyroid gland, spleen, pancreas, kidney, and bone marrow (46, 58, 61). Further supporting the role of other tissues as sites of viral replication, viral mRNA has been detected in bone marrow and PBMCs and lung, muscle, pancreas, kidney, colon, thyroid gland, gallbladder, omentum, and breast tissue (41, 46, 58, 60). Additionally, replication in the respiratory tract was suggested by the detection of high levels of TTV DNA in respiratory secretions (62–66).

In the lymphoid compartment, TTV shows a very broad cellular tropism as viral DNA can be detected in T and B lymphocytes, monocytes, and NK cells (45, 67–69). TTV tropism extends to granulocytes and other polymorphonuclear cells (45, 68). A recent study of the kinetics of TTV viremia following the administration of antilymphocyte immunosuppressive agents supports the hypothesis of T lymphocytes being the major site of replication (70).

EPIDEMIOLOGY

Distribution and Geography

Anelloviruses are widely distributed in both human and other mammalian populations throughout the world. The original surveys used detection methods based on primers that amplified the variable N22 region so that sequences similar to the prototype strain (TA278, genotype 1) are vastly overrepresented in the database of published sequences. Using primers based upon the conserved sequences in the UTR (Fig. 2), greater than 80% frequencies of viremia with TTV or related viruses have been found in virtually every population studied (49, 71–77). With the use of primers specific for individual genotypes or genogroups of TTV or those that differentiate TTV from TTMV sequences, high frequencies of coinfection with multiple variants of TTV have been described. In surveys of coinfections (78, 79), 84% to 89% of study populations (generally healthy adults) were viremic for TTV or TTMV, with coinfections of TTV and TTMV in 44% and frequencies of coinfection with multiple genotypes of TTV or TTMV of 40% to 70%. Infection with TTV-like viruses in pigs was similarly highly prevalent (33% to 100%) (10, 26, 27, 80–82).

Because of the near ubiquity of anelloviruses, the large number of genogroups and genotypes, and the difficulty in detecting coinfections, it is premature to conclude anything from previous investigations of possible geographical or risk group associations of genetic variants of these viruses.

Transmission

The detection of greater than 80% frequencies of viremia in adult populations indicates a considerable incidence of infection early in life. Infection with TTV occurs in the perinatal period; samples collected at birth are generally PCR-negative, with a rapidly rising prevalence of infection over subsequent months (83–87). Recent work with broader spectrum PCR assays (74, 77) and high throughput sequencing (66) demonstrate increasing detection frequencies of all three genera of human viruses in the early months and years of life. However, there are also conflicting reports of the detection of TTV DNA in cord blood, implying *in utero* infection (88, 89), a route that has also been suggested for porcine anellovirus transmission (90). During close follow-

up of a newborn child (91), high levels of TTV DNA were detected in the saliva from days 4 to 9 after birth, a period that coincided with a mild rhinitis. The TTV variant infecting the neonate was genetically identical to that present in the maternal saliva during the neonatal period. Although anecdotal, these findings are consistent with findings from other studies indicating the rapid acquisition of TTV infections around the time of birth. Anelloviruses are present at high concentrations in saliva (62–64) and other respiratory specimens (66) and are even detectable in exhaled breath (92). They may therefore be transmissible by kissing or other close contact between the child and parents or siblings.

There are likely other sources of anellovirus infection. Viruses are present in peripheral blood and have been demonstrated to be transmissible by parenteral routes such as blood transfusion (1, 93). However, this route is probably insignificant when compared to the rapid acquisition of anelloviruses in the perinatal period. Infectious virus (33) can be recovered from feces, and persistent shedding from this route has been documented (34), providing an additional potential source of oral transmission. The likely great stability of anelloviruses may lead to general environmental contamination.

PATHOGENESIS AND CLINICAL MANIFESTATIONS

Pathology

No specific pathology has been attributed to the replication of human anelloviruses in bone marrow, liver, or lymphoid tissue. The current best evidence of any pathological changes following primary infection with an anellovirus has come from experimental infections of pigs with torque teno sus virus (TTSuV). The inoculation of gnotobiotic piglets with plasma containing TTSuV1 resulted in transient thymic atrophy, membranous glomerulonephropathy, modest lymphohistiocytic infiltrates in the liver, and mild interstitial pneumonia (94). Similar results were observed following the infection of specific pathogen-free piglets with TTSuV2-containing liver homogenates, where the predominant pathological changes were interstitial pneumonia, membranous glomerulonephropathy, and inflammatory cell infiltration in portal areas of the liver (95).

The importance of viral genotype in relation to disease is poorly understood, and it may be that certain genotypes or species possess a greater pathogenic potential. While there is no current evidence to support this hypothesis in human infections, studies in pigs (96, 97) have suggested that infection with members of the TTSuV2 (genus *Kappatorquevirus*) may be more readily associated with pathology and coinfections than TTSuV1 (genus *Iotatorquevirus*) infections. Comparable examples of differences in disease associations of anelloviruses in humans may emerge in the future.

Immune Responses

Anellovirus infection is likely to be acquired around the time of birth (see Transmission), and this may lead to substantial immune tolerance as described for other viruses such as hepatitis B (HBV). Immunopathologic changes such as inflammation and lymphocyte infiltration are not observed in tissues where TTV replication occurs. The hypothesis that TTV and related viruses escape immunological detection is supported by the persistent nature of infection and the presence of multiple circulating genetic variants in plasma (see Genetic Variability) that may represent repeated rounds of reinfection. However, antibody reactivity to whole virions (98) or recombinant proteins expressed from ORF1 (99, 100) is frequently found in viremic and nonviremic individuals, and virions purified from plasma are often immune complexed with IgG (31). Despite this, there is no evidence at present for an association with or presence of persistent anellovirus infection in immune-complex deposition diseases such as glomerulonephritis.

Elevated TTV viral loads are routinely detected in patients with chronic congenital, acquired, or iatrogenic immunosuppression, indicating a potential role of the immune system in controlling viral replication (70, 101–111). In subjects undergoing immunosuppressive treatment for idiopathic pulmonary fibrosis (101) or following solid organ transplant (70, 103, 105, 106), a significant correlation between the degree of immunosuppression and the serum TTV titers was observed. However, one study demonstrated that the administration of a single immunosuppressant medication was sufficient to increase the TTV load significantly, and the addition of other immunosuppressive drugs did not further significantly increase the TTV load (102). In patients coinfected with HIV, viral loads of TTV (109–111) and TTMV (107) have been shown to have a significant negative correlation with CD4+ T-cell count despite the potential role of these cells as a major site of viral replication (70). Conversely, HIV-infected patients receiving highly active antiretroviral therapy (HAART) show a significant decrease in TTV vial loads following treatment (112, 113) as well as a decrease in the number of coinfecting TTV genogroups (114). Given this close correlation, the measurement of TTV viral load to be used as a clinical biomarker for immunosuppression has been suggested (115), although the clinical impact and usefulness of this approach remains to be determined.

Of great theoretical interest are the mechanisms of immune evasion that have evolved in TTV to establish persistent infection in immunocompetent individuals. One proposed mechanism of immune evasion is through the proinflammatory nuclear factor-kappaB (NF-kappaB) signaling pathway. Interactions between the ORF2 protein of a TTV isolate and subunits of the NF-kappaB signaling complex were shown to prevent nuclear entry of the p50 and p65 subunits, resulting in downstream inhibition of proinflammatory gene transcription (116). Predicted microRNAs (miRNAs) have been identified in a number of the available TTV sequences, although in none of the available TTMV or TTMDV genomes (117). Experimentally, the production of the miRNA encoded by the TTV-tth8 strain has been confirmed *in vitro*, and this has been shown to target the interferon-stimulated gene N-myc (and STAT) interactor (NMI), resulting in an inhibition of interferon signaling (117). Determining the function and targets of other anellovirus-encoded proteins and noncoding transcripts in the future could greatly enhance our current understanding of viral immune evasion and replication.

Disease Associations

As anellovirus infections are almost universally present in human populations, it is unlikely that any of these viruses are pathogenic *per se*. Based on the initial discovery of TTV in a posttransfusion hepatitis patient, the potential pathogenic role of this virus has been most extensively studied in relation to hepatic diseases. While some early studies suggested that the presence or viral load of TTV is related to various

hepatic disorders (118–124), numerous reports have been unable to substantiate these findings (125–129). Studies on the association of TTV with hepatitis are often complicated by coinfection with known hepatitis viruses (commonly HBV and hepatitis C [HCV]) or HIV. In light of the aforementioned correlation between viremia and immunosuppression, it is likely that a large proportion of reported increases in TTV prevalence or viral titer are a consequence rather than a cause of disease, and the presence of concomitant TTV infection has been shown to have no effect on the response of HCV infection to interferon treatment (130). Following interferon therapy, decreased TTV and SENV detection frequencies have been reported (131), although others have found that the decreases seen in TTV and TTMV titers following interferon (132) or TTV detection following combined interferon-ribavirin treatment (133) were transient.

Other investigations have concentrated on the potential roles of anelloviruses in childhood respiratory disease. High levels of TTV DNA are found in respiratory secretions and saliva (62–66), and the respiratory tract may thus be a major site of TTV replication. Infection with TTV coincided with a mild rhinitis in a neonate (91), and children hospitalized with acute respiratory disease showed higher TTV viral loads than controls (65, 73). Higher TTV viral loads were associated with impairment of lung function among asthmatic children, although frequencies of detection and viral loads were comparable to those of children without asthma (134). TTV titers have also been studied in other chronic lung diseases and have been correlated with airflow limitation in children with bronchiectasis (135) as well as in idiopathic pulmonary fibrosis (IPF) with primary lung cancer, where titers greater than IPF of lung cancer alone were seen (136). Again, whether these increases in viral titer are a result of disease progression or treatment rather than causal remains to be determined.

High levels of TTV replication in bone marrow have been suggested as being responsible for an otherwise unexplained case of aplastic anemia (137). Interestingly, the infection of chickens with the related CAV is associated with anemia and lymphopenia that is generally only clinically apparent in association with other viral coinfections, such as the Marek's disease virus. Interactions between TTV and herpesviruses have also been suggested in humans. The presence of TTV DNA in the skin of people with Kaposi's sarcoma lesions but not controls (138) suggests that the virus may be a cofactor in Kaposi's sarcoma-associated herpesvirus infections. It has also been reported that TTV isolates show significantly enhanced replication in Epstein-Barr virus (EBV)-converted Burkitt's lymphoma cell lines compared to EBV-negative controls (139).

LABORATORY DIAGNOSIS

PCR is the principal method used for the detection of anellovirus DNA in clinical specimens. Methods that use heminested or fully nested primers are capable of extremely high sensitivity and specificity. The UTR of TTV is much more conserved than coding regions and therefore more appropriate as a target sequence for PCR (60, 140, 141). PCR based on primers from regions around the transcription start site (Fig. 2) indicated that frequencies of anellovirus infections were close to universal, not only in humans but also in a range of nonhuman primates and other mammalian species (see Distribution and Epidemiology).

Because of the great genetic variability of anelloviruses, it has not been possible to date to develop effective genotyping assays for these viruses. A number of more recent methods employing rolling circle amplification have been reported. The use of these methods has allowed for greater sensitivity than PCR alone (142), and they have routinely been used for the generation of complete genome sequences in combination with random cloning or PCR using inverted primers designed within previously determined short sequences (12, 20, 143).

PREVENTION AND TREATMENT

As anellovirus infection is ubiquitous and still not convincingly associated with any clinical manifestations, measures to prevent transmission are unwarranted. Similarly, there is no current indication to treat infected individuals, although the treatment of coinfecting HIV or HCV infections has been shown to have an effect on anellovirus titers 12–114, 130–132).

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Hepatitis B Virus

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It has been over 40 years since the discovery of the hepatitis B virus (HBV), and yet the disease it causes remains a major public health challenge. Worldwide, over 240 million people have chronic hepatitis B (CHB), with the majority being in the Asia-Pacific region, and there are almost 800,000 deaths each year as a direct consequence of infection (2). HBV infection is the ninth leading cause of death worldwide. The main public health strategy to control hepatitis B infection for the last 30 years has been primary prevention through vaccination. According to WHO, as of 2013, more than 180 countries have now adopted a national policy of immunizing all infants with hepatitis B vaccine. However, a strategy of secondary prevention is clearly necessary to reduce the risk of long-term complications (cirrhosis, liver failure, and hepatocellular carcinoma) in those individuals who have CHB. The risk of these complications is strongly associated with persistent high-level HBV replication (3–5). Antiviral agents active against HBV are available. The long-term suppression of HBV replication has been shown to prevent progression to cirrhosis and reduce the risk of hepatocellular carcinoma (HCC) and liver-related deaths. However, currently available treatments fail to eradicate the virus in most of those treated, necessitating potentially lifelong treatment. WHO has set targets for both morbidity and mortality. A cure for CHB remains elusive, and a significant research effort is now being directed toward this goal.

VIROLOGY

Classification

The HBV is an enveloped 3.2-kb double-stranded DNA virus and prototypical member of the family *Hepadnaviridae*. HBV can be classified into ten major genotypes (A to J) based on nucleotide (nt) diversity of 8% or more over the entire genome (6–10). These genotypes show distinct geographical distributions (Table 1). As genotype designation is now based on the entire genomic sequence, it is a more reliable classification than the earlier serological subtype nomenclature (*adw*, *adr*, *ayw*, and *ayn*) used previously, which was based on the immunoreactivity of particular antibodies to a limited number of amino acids in the envelope protein. Particular HBV genotypes have unique insertions or deletions. For example, HBV genotype A varies from the other genotypes by an insertion of six nucleotides at the C-terminus of the core gene (11). HBV genotype D has a 33-nt deletion at the N-terminus of the Pre-S1 region (11). HBV genotypes E and G have a 3-nt deletion also in the N-terminus of the Pre-S1 region. HBV genotype G also has a 36-nt insertion in the N-terminus of the core gene (8), and the precore/core region has two translational stop codons at positions 2 and 28 and results in a HBeAg-negative phenotype. A very large number of subgenotypes (of < 8% but greater than 4% nt diversity) have been described in a number of these genotypes (A, B, C, D, and F), and recombination between two HBV genotypes has also been reported for genotypes B and C (12) and genotypes A and D (13), generating even more diversity.

Important pathogenic and therapeutic differences appear to exist among HBV genotypes (14, 15), including significant differences in intra- and extracellular expression of viral DNA and antigens (16). Genotype C is associated with more severe liver disease than is genotype B in Taiwan, while in India, genotype D is associated with more severe liver disease than is genotype A (17–20).

Virus Structure: Virion and Subviral Particles

Three types of virus-associated particles are typically present in the blood of HBV-infected persons. The complete infectious virus is spherical with a diameter of 42 nm. Negative-staining electron microscopy usually reveals a double-shelled structure (Fig 1A). The outer shell, or envelope, is formed by hepatitis B surface antigen (HBsAg) proteins, while the inner shell with a diameter of 27 to 32 nm is the viral nucleocapsid or hepatitis B core antigen (HBcAg). The viral nucleocapsid encloses the viral DNA and endogenous DNA polymerase. The sera of viremic patients also contain large numbers of two types of noninfectious particles: spherical particles 17 to 25 nm in diameter and filamentous forms 20 nm in diameter with a variable length. Both types of subviral particles are composed of HBsAg and are not infectious.

Genome Organization and Viral Proteins

The genome of HBV is a circular, partially double-stranded relaxed circular (21) (RC) DNA molecule. The two linear DNA strands are held in a circular configuration by a 226-base-pair (bp) cohesive overlap between the 5′ ends of the

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TABLE 1 Overview of the ten genotypes of HBV

Genotype	Subtype	Genome length (nt)	Frequency of mutation[1]		Global distribution
			PC	*BCP*	
A	A1	3221	Uncommon	Common	Africa, Asia
	A2				Northern Europe, North America
B	B1	3215	Common	Uncommon	Japan
	B2				Rest of Asia
	B3				Indonesia, China
	B4				Vietnam, Cambodia
C	C1	3215	Common	Common	Far East
	C2				Far East
	C3				Polynesia
	C4				Australian Indigenous Population
	C5				Philippines
D	D1	3182	Common	Common	Europe, Middle East, Egypt, India, Asia
	D2				Europe, Japan
	D3				Europe, Asia, South Africa, USA
	D4				Australia, Japan, Papua New Guinea
E		3212	ND	ND	Sub-Saharan Africa, UK, France
F	F1	3215	Uncommon	ND	Central and South America, Bolivia
	F2				Brazil, Venezuela, Nicaragua
	F3				Venezuela, Panama, Columbia
	F4				Argentina, Bolivia, France
G		3248	Very common	ND	USA, Germany, Japan, France
H		3215	ND	ND	USA, Japan, Nicaragua
I		3215	-	-	Vietnam, Cambodia
J		3182	-	-	Japan

Adapted from Locarnini et al. (650).
Note that genotype J has yet to be verified, as only one case has been isolated.
[1]PC, Precore mutations such as G1896A; BCP, basal core promoter mutations such as A1762T, G1764A; very common (most isolates); common (up to 50% of isolates); uncommon (less than 10% of isolates); ND, not described.

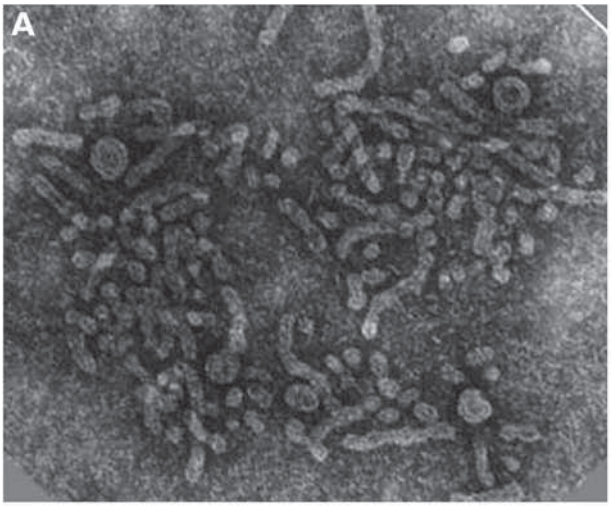
two DNA strands that contain 11-nt direct repeats called DR1 and DR2 (22). All known complete HBV genomes are gapped and circular (Fig 1B), comprising between 3181 and 3221 bases depending on the genotype (Table 1). The minus strand is not a closed circle and has a gap between its 3′ and 5′ ends. The viral polymerase is covalently bound to the 5′ end of the minus strand. The 5′ end of the plus strand contains an 18-base-long oligoribonucleotide, which is capped in the same manner as mRNA (23). The 3′ end of the plus strand is not at a fixed position, so most viral genomes contain a single-stranded gap region of variable length ranging from 20 to 80% of genomic length that can be filled in by the endogenous viral DNA polymerase.

The minus strand encodes four open reading frames (ORF), the longest of which encodes the viral polymerase (Pol ORF) (Fig 1B). The envelope ORF (Pre-S1, Pre-S2, and S) is located within the Pol ORF while the precore/core (Pre-C/C) and the X ORFs overlap partially with it. The ORFs overlap in a frameshifted manner and so the virus strand is read one and one-half times. The transcriptional template of the virus is the covalently closed circular (ccc) DNA, which exists in the cell nucleus as a viral minichromosome. From this template four major RNA species, the 3.5-, 2.4-, 2.1-, and 0.7- kb viral RNA, are transcribed (Fig 2A). The enhancer II/basal core, large surface antigen (Pre-S1), major surface antigen (S), and enhancer I/X gene promoters direct the expression of these four transcripts, respectively (22).

Pol ORF

The *Pol* gene is the longest ORF, spanning almost 80% of the genome and overlapping the other three ORFs. The Pol protein is translated from pregenomic RNA (Fig 2B). The 834 to 845 codons found in the Pol ORF have sequence homology to reverse transcriptases (22), and most parts of the ORF are essential for viral replication. The 90-kDa product of the Pol ORF is a multifunctional protein that has at least four domains (22) (Fig 2B). The N-terminal domain encodes the terminal protein that is covalently linked to the 5′ end of the minus strand of virion DNA. This part of Pol ORF is necessary for priming of minus-strand synthesis. An intervening domain with no specific recognized function is referred to as the spacer or tether region. The third domain encodes the RNA- and DNA-dependent DNA polymerase activities; that is, the reverse transcriptase. The C-terminal domain encodes ribonuclease (RNase) H activity that cleaves the RNA in the RNA-DNA hybrids during reverse transcription.

The terminal protein's role in priming reverse transcription includes the provision of the substrate tyrosine at amino acid 63 of the HBV Pol for the formation of the covalent bond between the enzyme and the first nucleotide (G) of the minus-strand DNA (24). The DNA polymerase domain contains the sequence motif tyrosine-methionine-aspartate-aspartate (YMDD), which is essential for reverse transcriptase activity (25) (Fig 2B). The RNase H domain, besides degrading the RNA template, also plays a role in viral RNA



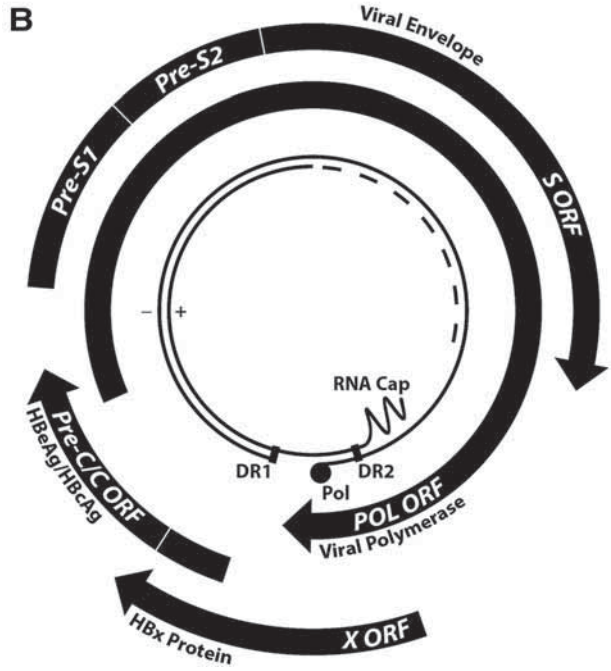


FIGURE 1A Electron micrography of the various forms found in the blood of HBV-infected persons. The 42-nm virions, both full and empty, can be seen. Within the empty particles, the 27 to 32 nm core structure can be visualized. The excess 22 nm subviral particles and filamentous forms of HBsAg are also present.

FIGURE 1B The circular double-stranded DNA genome of HBV showing the four main open reading frames (ORFs). The minus (-) and plus (+) DNA strands are marked. The HBV Pol and capped mRNA oligomer at the 5′ end of the (-) and (+) strands, respectively, as well as DR-1 and DR-2 are shown. The space between DR-1 and DR-2 is the "cohesive overlap region." The plus strand is typically incomplete.

packaging, in optimizing priming of minus-strand DNA synthesis and in elongation of the minus-strand viral DNA.

The Pol encodes at least two T-cell epitopes within its catalytic domains at amino acid residues 107 to 115 and 227 to 235 (26). Changes in these epitopes have been related to successful interferon-α therapy and viral clearance during acute infection (27).

Pre-C/C ORF

The Pre-C/C ORF encodes the core protein P21, which is the major polypeptide of the nucleocapsid and expresses the HBcAg. The HBc protein is 183, 185, or 195 amino acids long, depending on the genotype of the virus (Table 1). ORF C is preceded by a short, upstream, in-phase ORF called the Pre-core region from which the soluble hepatitis B early antigen (HBeAg) is made (22).

HBc Protein

The HBc protein has two distinct domains with amino acid residues 1 to 144 required for the assembly of the 32-nm nucleocapsid, while the arginine-rich, C-terminal residues from around 140 onward form a protamine-like domain that mediates nucleic acid binding and is involved in viral encapsidation and DNA replication (22). This arginine-rich region contains four clusters and includes a potential nuclear localization sequence. The core protein contains many hydrophilic and charged amino acids, and, when expressed, it becomes phosphorylated (8). Phosphorylation of serine 170 to 172, between arginine clusters 3 and 4, appears to block nucleic acid binding and may negatively regulate nuclear localization of the core protein (8). The HBc protein is also translated from pregenomic RNA (Fig 2A) and carries the hepatitis B core antigen (HBcAg) epitopes, which are cross-reactive with the HBeAg epitopes (discussed below) (Fig 2C).

Aside from the function of HBcAg in the construction of the viral nucleocapsid, HBcAg may play an active role in regulating viral transcription. This was first suggested following experiments that demonstrated HBcAg aggregating in different cellular compartments depending on the phase of infection (28) (see Laboratory Diagnosis). The immunotolerant phase, in which high HBV-DNA levels are seen, is associated with a predominant nuclear distribution of HBcAg. In the immune clearance phase, HBcAg becomes predominantly cytoplasmic, in conjunction with decreasing viral load. HBcAg becomes undetectable in the immune control phase. HBcAg exhibits preferential binding for HBV-covalently closed circular DNA (cccDNA), modulating its transcription by altering nucleosomal packaging (29). These studies demonstrate the multifunctional nature of HBV proteins.

HBe Protein

The HBeAg is essentially a soluble secretory form of the HBc protein and is considered an accessory protein of the virus. The Pre-C sequence is upstream of core, and translation initiating from the Precore initiation codon produces the Pre-core protein that contains the entire Core protein sequence plus an additional 29 amino acids at the N-terminal end (P25) (22). The Precore protein is translated from the Pre-C/C mRNA (Fig 2A). The first 19 amino acids of the Precore protein form a secretion signal that allows the translocation of the Precore protein into the lumen of the endoplasmic reticulum (ER). These 19 amino acids are cleaved by a host-cell signal peptidase, leaving the Precore protein derivative P22. The P22 is then secreted through the ER and Golgi apparatus and further modified by C-terminal cleavage of up to 34 amino acids, resulting in the secretion of a heterogeneous population of proteins of approximately 17 kDa, serologically defined as HBeAg (22) (Fig 2A). Thus, HBe protein differs in almost all aspects from HBc protein, although the primary sequences of these two molecules are almost identical. Some of the HBe protein does not reach

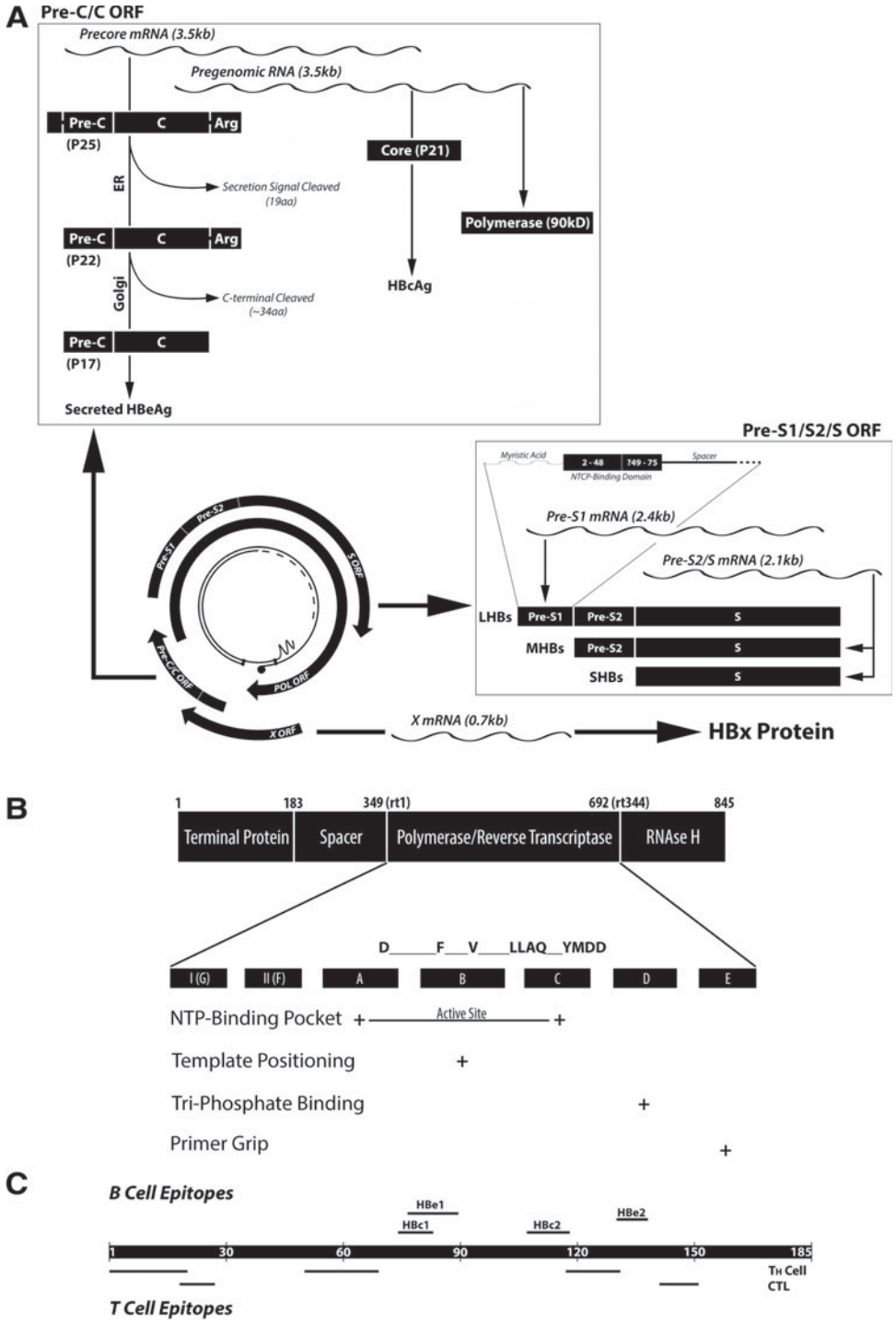


FIGURE 2A Biosynthesis of the precore/core, polymerase, envelope, and X proteins from the various HBV transcripts. The two major genomic 3.5-kb transcripts are the larger Precore mRNA, from which the Precore protein (HBeAg) is made, and the smaller pregenomic mRNA that encodes the core and polymerase and is the template for reverse transcription. The single 2.4-kb RNA makes LHBs, while the various 2.1-kb mRNAs translate MHBs and SHBs. The HBx protein is translated from the 0.7-kb mRNA.

FIGURE 2B The functional domains of the polymerase-reverse transcriptase of HBV See Bartholomeusz et al. (475) for a more detailed discussion.

FIGURE 2C Linear, schematic representation of the core protein amino acid residues and the immunodominant B cell and T cell epitopes. See text for details.

the ER lumen and is not cleaved at all. The P25 HBe protein also expresses a nuclear transport signal (8). Thus, HBe proteins of variable length are found in practically all compartments of the cell, as well as being secreted.

The HBe protein is essential for establishing persistent infection. Mutants of HBV without a functional Pre-C sequence and HBe protein are found commonly during chronic infection. An HBeAg-negative mutant of the woodchuck HBV is infectious for newborn woodchucks but is unable to establish persistent infection (30). Similar scenarios have been described for HBV (31), suggesting that the HBe protein may suppress the immune elimination of HBV-infected hepatocytes. It certainly can function as a tolerogen *in vivo* (32). HBeAg has been shown to slow HBV-DNA replication *in vitro* by reducing HBcAg dimerization, thereby reducing encapsidation of pgRNA (33). The HBeAg is not essential for HBV replication but is absolutely required for the establishment of persistent infection (34, 35). Expression of the precore protein has been shown to have anti-apoptotic properties *in vitro* (36), and *in vivo* it can act as an immune tolerogen (32). Thus, the HBeAg is an important accessory protein in the life cycle of HBV. Two major groups of mutations have been identified, which result in reduced or blocked HBeAg expression.

The first group of mutations affects the basal core promoter (BCP), typically at nt 1762 and nt 1764, resulting in a transcriptional reduction of the Pre-C/C mRNA (35). Mutations in the BCP, such as A1762T plus G1764A, may be found in isolation or in conjunction with precore mutations (see below), depending on the HBV genotype (Table 1). The double mutation of A1762T plus G1764A results in a significant decrease in HBeAg levels and has been associated with an increase in viral load. This pattern of mutation is found in genotype A-infected individuals as the major cause of HBeAg loss (35) and typically in association with the precore mutations in the other HBV genotypes (37). Importantly, these BCP mutations do not affect the transcription of HBV pgRNA or the translation of the core or polymerase protein. Thus, by removing the inhibitory effect of the precore protein on HBV replication, the BCP mutations appear to enhance viral replication by suppressing Pre-C/C mRNA relative to pregenomic RNA (35).

The second group of mutations includes HBV mutants with a translational stop-codon mutation at nt position 1896 (codon 28: TGG; tryptophan) of the precore gene. The single base substitution (G-to-A) at nt 1896 gives rise to a translational stop codon (TGG to TAG; TAG = stop codon) in the second last codon (codon 28) of the precore gene located within the structure of pgRNA. The nt G1896 forms a base pair with nt 1858 at the base of the stem loop. In HBV genotypes B, D, E, G, and some strains of genotype C, the nt 1858 is thymidine (T). Thus, the stop codon mutation created by G1896A (T-A) stabilizes the structure based on conventional Watson-Crick pairing (Fig 3). In contrast, the precore stop codon mutation is rarely detected in HBV genotype A, F, and some strains of HBV genotype C, as the nt at position 1858 is cytidine (C), maintaining the preferred Watson-Crick (G-C) base pairing. Other mutations have been found within the precore transcript that block HBeAg production, including abolition of the methionine initiation codon (37), and the stop codons within the precore/core region of genotype G.

The HBeAg has two B cell epitopes, one of which is linear (HBe1) and the other (HBe2) conformational. The HBe1 epitope is found at core amino acid positions 76 to 89, and the HBe2 epitope is at positions 130 to 138 (38) (Fig 2C).

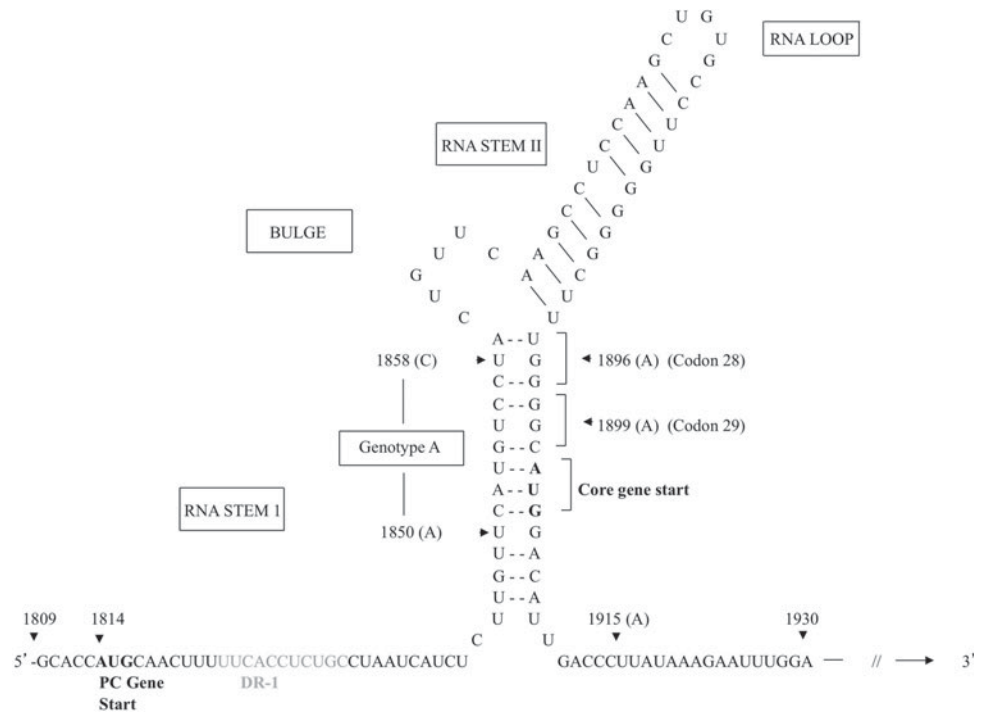


FIGURE 3 Diagrammatic representation of the epsilon (ϵ) stem-loop structure of HBV. This is a highly conserved structure within the 10 genotypes of HBV, and the positions of base changes for genotype A2 are shown, as are the common translational precore mutations at G1896A (precore stop codon: UAG) and G1899A.

Two immunodominant core epitopes (HBc), localized between residues 74 and 83 (HBc1) (38), are colinear with HBe1 and to residues 107 to 118 (HBc2) (39). Not surprisingly, HBcAg and HBeAg are highly cross-reactive at the T cell level (26, 40) (Fig 2C). Two cytotoxic T lymphocyte (CTL) epitopes of HBc can be mapped to residues 18 to 27 (human leukocyte antigen HLA-A2 restricted) (41) and residues 141 to 151 (HLA-A31 and HLA-Aw68 restricted) (42). Three T-helper cell (T_H) epitopes have been found on the core protein at amino acid residues 1 to 20, 50 to 69, and 117 to 131 (43).

Pre-S/S ORF

The HBsAg contains small (SHBs), medium (MHBs), and large (LHBs) proteins, all of which exist in two forms differing in the extent of glycosylation (Fig 2A). N-linked glycosylation and glucosidase processing are necessary for virion, but not subviral particle, secretion (8). The filaments consist of the same ratio of surface proteins as the virion envelope.

SHBs

The SHBs domain is 226 amino acids long and is the most abundant protein of all three HBV-associated particles. The SHBs is found in a glycosylated and a nonglycosylated form. It contains a high number of cysteine residues that cross-link with each other, forming a conformational loop that is the major antigenic determinant of HBsAg, the "a" determinant or antigenic loop (AGL) (44). The AGL is responsible for initiating viral entry to target hepatocytes by initially interacting with cell-surface heparan sulfate proteoglycans (HSPG) (45), which likely explains its presence on all known HBsAg isolates in a highly conserved fashion. The AGL has the subdeterminants "d" or "y" and "w" or "r." Determinant d has a lysine at position 122, and y is represented by an arginine; likewise, determinant w has a lysine at position 160 and r has an arginine (46). These antigenic subtypes elicit cross-protecting anti-HBs following immunization. The "a" determinant has been renamed the major hydrophilic region (MHR), spanning amino acids 99 to 169, and is considered the major neutralization domain for anti-HBs.

MHBs

The MHBs Pre-S2 domain is a minor component of the virion or HBs particle and consists of the S and a 55-amino-acid N-terminal extension (22). It is either singly- or doubly-glycosylated but is not essential for virus assembly and release. The immunogenic epitopes are not conformationally dependent, as is the case for SHBs.

The central part of the Pre-S2 domain carries the major antigenic epitope, and the region between amino acids 3 and 16 has the ability to bind polymerized human serum albumin (8). The significance of this interaction is unknown, but many utilize albumin as an acting co-receptor or to mask the immunogenicity of this epitope (47). The MHBs is considerably more immunogenic than SHBs at the B cell level (48), and Pre-S2-containing HBs particles generated from animal cell lines have been used in some countries as a prophylactic vaccine (49).

LHBs

The LHBs contains a further N-terminal extension to the M protein of 108 or 119 amino acids (depending on the subtype/genotype) (Table 1) and is more prevalent than MHBs in virions and filaments but less prevalent in HBs spheres. Thus, the LHBs contains three domains, Pre-S1, Pre-S2, and S, and is glycosylated. In mature virions and HBs particles, the Pre-S1 domains are exposed on the surface, and both the S domains and parts of the Pre-S2 sequence are covered by the Pre-S1 sequence of LHBs (8). In contrast to MHBs, the LHBs is essential for infection and viral morphogenesis, as the Pre-S1 region encodes the NTCP-binding domain (see Viral Attachment) (50, 51). The N-terminal end of the Pre-S1 domain is myristoylated at glycine 2, a function that is not required for virion formation and release but is essential for the ability of the virus particle to interact with the cellular plasma membrane (51). Amino acids 2 to 48 of the Pre-S1 sequence specifically interact with NTCP and contain a highly conserved motif [9-NPLGF(F/L)P-15] that is crucial for binding (Fig 2A). Residues 49 to 75 are also required for infection, but the precise function of this region is not known (51).

The LHBs has important antigenic sites for both B and T cells that appear to play critical roles in recovery from viral infection or protection from infection (52). The major immunogenic epitopes within the Pre-S1 coding region are at amino acids 27 to 35, 72 to 78, and 95 to 107. LHBs is also highly immunogenic for T cells in humans, at residues 21 to 48 as well as 81 to 108.

X ORF

The X ORF encodes a 154-amino-acid polypeptide (HBx) of 17 kDa (Fig 2A). This second accessory protein of HBV is conserved in a similar form across all the mammalian hepadnaviruses. The expression of full-length HBx protein is dispensable for virus replication *in vitro* but is a critical component of the infectivity process *in vivo* (53). HBx behaves as a transcriptional transactivator of a number of viral and cellular gene promoters through direct interaction with transcription factors, such as the RPB5 subunit of RNA polymerase II, TATA-binding protein, and the ATB domain, and is also involved in the activation of signal-transduction pathways, such as the Ras/Raf/MAP kinase cascade (8). HBx also increases the transcription of the cccDNA template by modifying its epigenetic regulation (54). In brief, epigenetic modification controls how tightly wound chromatin is to structural histone proteins. More tightly wound chromatin is not translated, whereas the converse is true; histone acetylation loosens chromatin but methylation tightens it. HBx recruits histone acetyltransferase and deacetylase enzymes in a fashion designed to prevent hypoacetylation (55). On the other hand, HBx *in vitro* inhibits protein arginine methyltransferase 1 (PRMT1), which functions to increase histone methylation (56).

Another important target of HBx is the ubiquitin-proteasome system (UPS), which is a conserved cellular pathway involved in protein ubiquitination and proteolysis (57). Many enzymes and proteins contribute to the function of this pathway, but of interest in the context of HBx is the damaged DNA binding protein-1 (DDB1) and the Cullin4 (CUL4) protein. HBx has been shown to bind DDB1, integrating itself into the DDB1-CUL4 complex, thus allowing regulation of its function (58). The importance of this interaction with regards to HBV replication has been demonstrated *in vitro* and in woodchuck hepatitis virus (WHV) models (59–61), but other consequences are as yet unknown.

The HBx protein also has a role as a cofactor in HBV-mediated carcinogenesis (62). The exact mechanism(s) whereby HBx contributes to the development of HCC is

unknown, although HBx-associated transactivation activity leads to alterations in cellular gene expression that contributes to transformation (63). Findings in support of this hypothesis include:

- HBx suppression of p53-mediated upregulation of microRNA pathways that enhance the growth and metastatic potential of tumors in a mouse HCC model (64).
- Studies of epigenetic changes show that HBx induces hypermethylation of genes with tumor suppressing activity (65).
- Disruption of signaling pathways critical to maintenance of cellular homeostasis (66).
- Abrogation of p53-dependent apoptosis and cell cycle checkpoint deregulation (67).
- HBx targeting of the 26S proteasome complex has also been implicated in hepatic carcinogenesis (57, 68, 69), suppressing viral antigen processing, and, consequently, presentation, thereby assisting evasion of immune detection (70).

Viral Replication

The replication cycle of HBV revolves around two key processes:

1. Generation of HBV cccDNA from genomic RC DNA and its subsequent processing by host enzymes to produce viral RNA; and
2. Reverse transcription of the pregenomic (pg)RNA within the viral nucleocapsid to form RC DNA, completing the cycle (Fig 4). Earlier and later events are described below in the context of these two processes. The detailed molecular biology of HBV replication has been thoroughly detailed previously (71) and will not be repeated here.

Attachment and Penetration

The first stage of infection is attachment to a susceptible hepatocyte and penetration of HBV into the cell cytoplasm following the binding of the HBV envelope to its specific cellular receptors. The "a" determinant region of the LHBs component of the HBsAg attaches to hepatocyte-associated heparan sulfate proteoglycans (HSPG) as the initial step in cellular entry (Fig 4) (72). This facilitates the next binding step of LHBs to the protein complex that forms the receptor for HBV, the sodium taurocholate cotransporting polypeptide (NTCP), which normally functions to maintain bile acid homeostasis via the enterohepatic circulation (50, 73). NTCP is encoded by the human *SLC10A1* gene, which is found on the long arm of chromosome 14. The pre-S1 domain of the HBV envelope protein contains two regions within the 75 amino acids at the N-terminal end, which are responsible for binding to NTCP (50). Myristoylation of these sequences is essential for virion infectivity (74, 75). Subsequent receptor-mediated endocytosis is responsible for the delivery of viral nucleocapsids into the cytoplasm. The possibility of utilizing drug therapy to block viral entry has been demonstrated *in vitro* using natural substrates of NTCP to inhibit viral infection of cell lines (50, 76, 77) (see Novel Therapeutic Strategies).

Conversion of Genomic RC DNA into cccDNA and Transcription of the Viral Minichromosome

Intracellular viral nucleocapsids are transported to the nuclear membrane where they uncoat (78) and deliver their genetic load into the nucleus. The genomic RC DNA is released and then converted into cccDNA using host cell enzymes resulting in the formation of the viral minichromosome, the template of HBV that is used for the transcription of all the viral mRNAs (79, 80) (Fig 4). The first step in this conversion, the release of the Pol protein from the 5′ end of the minus strand is achieved by utilizing the host DNA repair enzyme tyrosyl-DNA-phosphodiesterase 2 (TDP2) (81). The subsequent steps in cccDNA generation are yet to be elucidated.

Two classes of transcripts are synthesized from the HBV minichromosome utilizing host cell RNA polymerase II, genomic length and subgenomic length (22). Both classes contain heterogeneous transcripts that are of positive orientation, are capped at the 5′ end, and polyadenylated at the 3′ end. The subgenomic transcripts function exclusively as mRNAs for translation of the envelope (Pre-S1, Pre-S2, and S proteins) and X proteins. The two genomic transcripts are longer than genomic length and encode the Pre-C, Core, and Pol ORF. Generation of the Pre-C/C mRNA is an early transcription/translation event and functions in translation of the precore protein, which is processed and then secreted as HBeAg (22). The Pre-C/C mRNA is not involved in reverse transcription. In contrast, the pregenomic RNA is multifunctional, serving as the template for reverse transcription into the viral (-) DNA strand and for translation of both HBcAg and HBV Pol.

HBV Genomic Replication via Reverse Transcription

HBV genomic replication is initiated with packaging of the pgRNA and the newly translated viral polymerase/reverse transcriptase (HBV Pol) into subviral core particles, forming replication complexes. Reverse transcription occurs within the HBV nucleocapsid. As the HBV Pol is being translated off the same pgRNA molecule that will be packaged, the N-terminal region (terminal protein) specifically binds to the "bulge" region of a unique RNA stem loop structure, known as epsilon (ϵ), at 5′ end of the pgRNA (82) (Fig 3). This RNA structure also acts as the encapsidation signal around which cytoplasmic core protein dimers assemble into nucleocapsids. The HBV Pol undergoes a conformational change, which results in enzymatic activation, with the terminal protein domain priming DNA synthesis (24). This Pol-oligonucleotide (Pol-G-A-A) complex then translocates to the complementary sequences of a direct repeat (DR-1) region located at the 3′ end of the pgRNA. Minus (-) DNA strand synthesis then continues until it reaches the 5′ end of the pgRNA molecule (23), generating a short terminal redundancy of approximately 8 to 9 nt. While reverse transcription is proceeding, the pgRNA is degraded by the RNaseH activity of the HBV Pol, except for the 5′-capped terminal 18 nt fragment that contains the DR-1 sequence (22). This fragment includes a 6-nt homology to the direct repeat sequence, which allows circularization of the (-) DNA strand. The 18-nt capped RNA structure is then translocated to a second DR sequence (DR-2) on the 5′-end of the newly made (-) DNA strand, where it acts as primer for (+) strand synthesis, using the (-) strand as a template (23) (Fig 4). Synthesis of the (+) strand DNA continues until it reaches about 50 to 70% of the length of the (-) strand.

Virion Assembly and Release

The viral envelope, the small particles, and filaments are synthesized and assembled at the ER membranes and then bud into its lumen. The HBc protein is synthesized in the

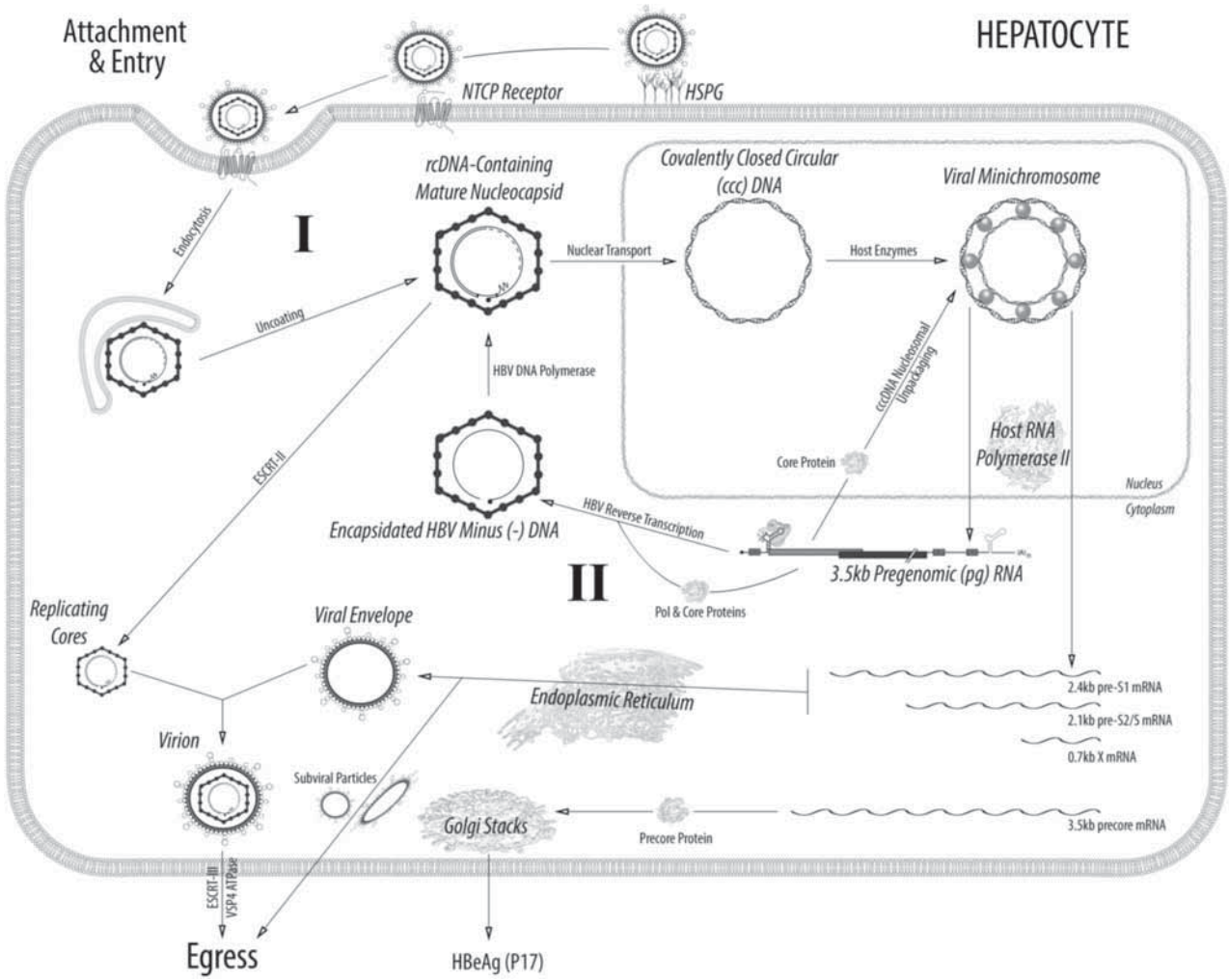


FIGURE 4 Life cycle of HBV. Following attachment, penetration, and uncoating, the viral nucleocapsid is released into the cytosol and transported to the nuclear pore. The relaxed circular DNA is delivered into the nucleus where it is converted into cccDNA, and the viral minichromosome is generated. Transcription of the viral minichromosome produces the genomic and subgenomic HBV mRNA transcripts. Translation of the pregenomic RNA in the cytosol produces the Core and Pol proteins, and, in association with Hsp-60, all are selectively packaged into a replication complex. Within the nucleocapsid, reverse transcription begins. Synthesis of the (+) DNA strand proceeds from the RNA primer to the 5′ end of the (-) DNA strand (23). The short terminal redundancy of the (-) DNA strand permits the transfer of the 3′ end of the growing short (+) strand from the protein-linked 5′ end to the 3′ end of the minus strand, thereby circularizing the genome and allowing continuation of DNA synthesis, generating the genomic RC DNA molecule with the HBV Pol covalently attached to the 5′ end of the (-) DNA strand. The envelope proteins Pre-S1, Pre-S2, and S are translated at the rough endoplasmic reticulum (ER) and then bud into the lumen of the intermediate compartment. Approximately 50% of the Pre-S1-enriched ER-membrane areas envelope core particles. The HBV virions and subviral particles are then secreted into the extracellular space by usurping the cellular ESCRT pathway. The nucleocapsids can also be transported to the nucleus via an intracellular conversion pathway, thereby increasing the number of nuclear cccDNA molecules.

cytosol and assembled independently of the envelope proteins (22). Immature nucleocapsids containing pgRNA-Pol can only commence envelopment once the nucleocapsid is phosphorylated. This latter process is coupled to the initiation of (-) strand synthesis (22). The assembled nucleocapsids containing genomic RC DNA are then selectively enveloped before exiting the cell (22). A further bias exists in favor of the export of genomes that have completed (-) strand DNA synthesis and at least started (+) strand synthesis (22). Correct assembly of replicating cores with the viral envelopes requires a critical relative molar ratio of Pre-S1 to S. Insufficient S or excessive Pre-S1 production results in abnormal assembly and release (22). As with many other enveloped viruses, HBV usurps the endosomal sorting complex required for transport (ESCRT) network of proteins (Fig 4), which is a cellular network normally involved in the formation of multivesicular bodies and daughter cell cytokinesis (83–85). Various components of this system have important roles in the HBV replication cycle. In particular, the formation of mature nucleocapsids requires the ESCRT-II protein (83), and virion budding and egress require ESCRT-III and the VPS4 ATPase (85).

Variant Viruses

The replication strategy of HBV is essentially error prone. This poor fidelity is a result of two factors. First, there is a

lack of a proofreading or editing mechanism for the HBV Pol. Secondly, all reverse transcription processes have an intrinsic G-to-A hypermutation rate largely attributable to host APOBEC enzymes, and hepatitis B is no exception (86). Up to 10^7 base-pairing errors may occur each day (87). The rate of HBV virion production is approximately 10^{11} virions per day. The error rate of the HBV Pol has been calculated as 10^{-7} per nucleotide per day (88). Therefore, each day, 10^{14} nucleotides (10^{11} virions x 10^3 nt) are replicated with potentially 10^7 base pairing errors (87). Thus there is the potential for every nucleotide in the genome to be substituted daily. Although mutations can occur randomly along the HBV genome, the overlapping open reading frames constrain the evolution rate, limiting the number and location of viable mutations. Because chronic HBV infection frequently persists for decades, many variants exist within the one host at any given time; HBV therefore exists as a quasispecies, with one variant typically dominating. There are three forms of variant HBV that are encountered commonly in clinical practice:

a. Precore and basal-core promoter variants, which have an HBeAg-negative phenotype, typically emerge after HBeAg seroconversion and are the most common cause of HBeAg-negative CHB (discussed above).
b. Envelope mutants that have been selected by hepatitis B immunoglobulin therapy in the posttransplantation setting or by vaccines following prophylactic immunization.
c. Antiviral resistant polymerase mutants that emerge in the setting of nucleos(t)ide analogue (NA) therapy are responsible for virological breakthrough and treatment failure (discussed below).

Envelope Gene Mutations

Viral genomes that cannot synthesize Pre-S1 and Pre-S2 proteins have been found to occur frequently and are often the dominant virus populations in patients with CHB (89). Pre-S1 mutants are associated with intracellular retention of the viral envelope proteins and the classical histological appearance of type I ground glass hepatocytes (GGH) (90). Type II GGH indicates the presence of Pre-S2 mutants. The Pre-S2 region overlaps the spacer region of the Pol protein, which is not essential for enzyme activity; thus both envelope mutants replicate in their infected host but with differing histopathological sequelae. Presence of GGH has been associated with increased hepatocarcinogenic potential, in part, attributable to a cellular growth advantage conferred by enhanced expression of vascular endothelial growth factor-A (91).

Current hepatitis B vaccine contains the major HBsAg. The subsequent anti-HBs response to the major hydrophilic region (MHR) of HBsAg between residues 137 and 148 induces protective immunity (51). Mutations within this epitope have been selected during prophylactic vaccination (44) and following treatment of liver transplant recipients with hepatitis B immune globulin (HBIG) prophylaxis (92). Most vaccine-HBIG escape isolates have a mutation from glycine to arginine at residue 145 of HBsAg (sG145R) or aspartate to alanine at residue 144 (sD144A). The sG145R mutation has been associated with vaccine failure (44) and has been transmitted, resulting in persistent infection and disease. Other important mutations have been found at a MHC class I-restricted T-cell epitope in HBsAg between codons 28 and 51 (93).

EPIDEMIOLOGY

Incidence/Prevalence

Approximately 240 million people have CHB (2). HBV is endemic in Asia, sub-Saharan Africa, Micronesia, and Polynesia, as well as in indigenous populations in Alaska, Northern Canada, Greenland, Australia, New Zealand, and within population groups in South America and the Middle East (94–96) (Fig 5). In developed countries, CHB is also seen in specific groups with percutaneous and sexual-transmission risk factors. These include injecting drug users, dialysis patients (3% to 10%), men who have sex with

FIGURE 5 Global prevalence of HBsAg, WHO clarifies areas of high, medium, or low endemicity for HBV if the prevalence rates of HBsAg are ≥8%, 2 to 7%, or <2% respectively. (Adapted with permission from Ott et al. [649]).

men (6%), and people with multiple sexual partners. In low-prevalence countries, such as the USA, Canada, and Northern Europe, the prevalence of HBV infection is 0.1% to 2%. In intermediate prevalence countries (Mediterranean, Central and South America, Japan, and the Middle East) 3 to 5% of the population is infected and a 10-to-20% infection rate in high prevalence regions, such as Southeast Asia, Africa, China, and sub-Saharan Africa (95).

The prevalence of HBeAg-negative CHB is increasing worldwide. Rates of 50 to 80% in India and the Mediterranean area (Italy, Greece, and Israel) and 40 to 55% in East Asia (Hong Kong, Taiwan, and Japan) have been reported (34,97–102).

Transmission

HBV antigens can be detected in almost all secretions (103–105), but only blood, saliva, and semen have been shown to be infectious (106, 107). The virus can be transmitted by perinatal, percutaneous, and sexual exposure or via close person-to-person contact in the presence of open cuts and sores (a common transmission method in children). However, the commonest route of transmission worldwide is perinatal infection (108).

The mode of transmission of HBV infection varies geographically. In high-prevalence areas, such as Southeast Asia and China, perinatal and early childhood horizontal transmission are the most common, resulting in high levels of chronicity (95% perinatal, 30% before 5 years). Perinatal transmission is likely to occur at birth or in the neonatal period; given the efficacy of neonatal vaccination, *in utero* infection is rare. The primary determinants of transmission are HBeAg status and HBV DNA level in the mother. The vertical transmission rate in HBeAg-positive women is 90% without intervention, compared to 32% for HBeAg-negative women (109). The failure rate of immunoprophylaxis in infants born to HBeAg-positive women approaches 10% when the HBV DNA level is $>10^8$ IU/ml (>7 $\log_{10}$ copies/ml) but is about 3% when the HBV DNA level is below this threshold (1).

In low prevalence areas, including the United States, Canada, Australia, and western Europe, sexual and percutaneous spread (primarily injecting drug use) is also seen. Limited data are available to describe risk per exposure; however, it has been observed that heterosexual partners of HBsAg positive individuals have a 25 to 59% seroprevalence rate (110). In the setting of needlestick injury sustained by healthcare workers, the occurrence of HBV transmission is rare since the implementation of universal vaccination in this special risk population (111).

In the setting of exposure during adulthood, HBV infection typically manifests as acute hepatitis (see below). The progression to chronic hepatitis is rare in the immunocompetent adult, occurring in $<1\%$ of individuals (112). Patients already infected with HIV, or immunosuppressed for other reasons, are at increased risk of chronicity after acute hepatitis B (113). The natural history of adult-acquired chronic infection differs from perinatal disease, with a higher rate of spontaneous and treatment-induced HBsAg seroconversion and lower rates of progression. However, the largest disease burden in most developed countries remains perinatal or childhood-acquired disease in immigrants from endemic areas (114).

Uncommon Modes of Transmission

Transfusion-related hepatitis B is now uncommon in countries where blood is obtained from unpaid donors screened for HBsAg and anti-HBc, with an estimated risk in the US of 1 per 3.2 million donors (115).

Nosocomial HBV transmission still occurs despite the availability of vaccination and postexposure prophylaxis, although HBV infection in healthcare workers (HCW) has declined dramatically in countries with HCW vaccination programs (111). Transmission from HCW to patient is rare but reported. The commonest situations are patient-to-patient or patient to HCW via needlestick injuries (syringe, suture needles) or scalpel. Nurses, dialysis staff, surgeons, dentists, and their assistants are at highest risk. Incomplete vaccination of staff, failure to apply universal precautions, and incorrect needle disposal technique are the commonest reasons for transmission. HBeAg positivity is associated with a higher risk of transmission, although transmission has been reported from HBeAg-negative individuals (116), confirming that HBV DNA levels are a better marker of infectivity.

Transmission from any organ donated by HBsAg positive donors is well recognized, and all donors are screened for HBsAg. Anti-HBc-positive liver donors can also transmit infection. This is less common in renal and other transplants (117).

PATHOGENESIS

HBV is generally noncytopathic, and the liver inflammation and secondary fibrosis that complicate infection are primarily immune-mediated. The complex interplay that occurs between the host and pathogen in the setting of HBV infection is now starting to be unraveled (Fig 6). The experimental study of HBV immunopathogenesis has been limited by the absence of suitable animal models and *in vitro* cell lines permissive for HBV infection. HBV can infect chimpanzees but typically causes a self-limiting acute hepatitis, different in severity and outcome to human disease. This model is resource intensive and no longer ethically permitted. Surrogate small animal models include the infection of ducks with duck hepatitis B virus (DHBV) and the woodchuck with woodchuck hepatitis B virus (WHBV). These models have been useful for studies of molecular virology but less so for pathogenesis work. Transgenic mouse models have allowed for a better understanding of the role of cytokines and noncytolytic viral clearance in the pathogenesis of HBV but not the humoral immune response.

Acute Infection

In contrast to hepatitis C virus, HBV does not start to replicate efficiently immediately post inoculation (118). Instead, experimental data, mainly from animal models, but also in humans, show that a "lag period" of 4 to 7 weeks is observed before HBV DNA and HBV antigens become detectable in the serum or liver. The explanation for this lag phase remains unclear. In acute HBV infection of chimpanzees, gene-array studies (119) found that no interferon-related genes were activated within the liver during the lag phase, despite a subsequent adaptive immune response then clearing HBV in all animals. While this work suggested that HBV might be a stealth virus that evades early innate immunity, increasing evidence suggests that HBV may be detected by the innate immune system almost immediately, which suppresses viral replication early after infection. This suppressive response includes the recruitment of NK/NKT cells (118), secretion of cytokines IFNγ and TNFα, interleukin-6, -8, and -1β from Kupffer cells (120), and Toll-like receptor (TLR) signaling, especially within the hepatocyte, in early HBV infection (121–123). However, HBV

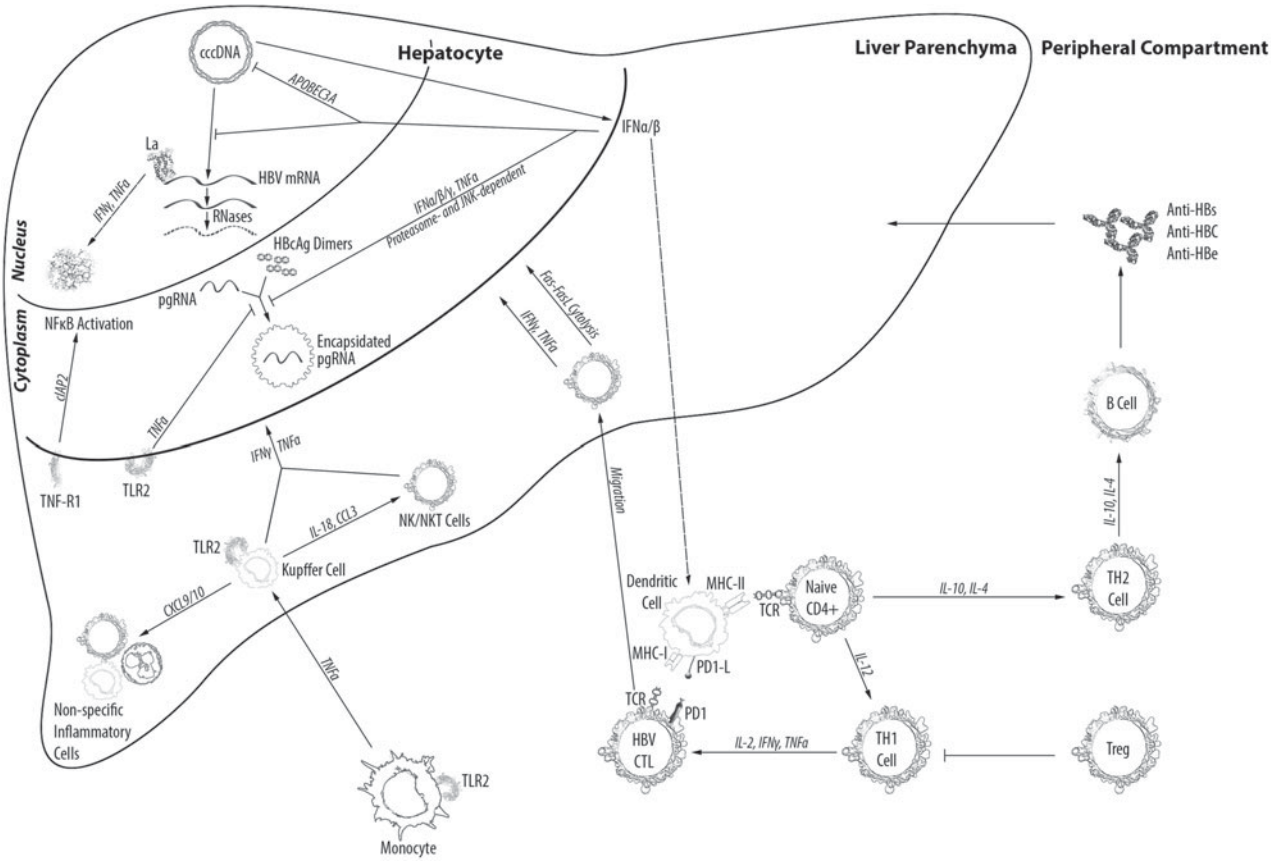


FIGURE 6 The host immune response directed against HBV requires coordinated action of both innate immunity and cellular and humoral adaptive immunity to affect both noncytolytic and cytolytic activity. The pathways of noncytolytic clearance are highlighted (see section "Pathogenesis").

has evolved to respond by downregulating host innate immune responses at the IFNα level (124).

Once HBV DNA becomes detectable, replication increases exponentially to peak at levels of 10^8 to 10^9 copies/ml (125). In adults who can control the virus, viral replication then declines, preceding the onset of clinical hepatitis (126). Acutely infected chimpanzees also experience a rapid drop in viral replication before any detectable cellular infiltration or liver injury occurs (127). This phenomenon is explained by a process of noncytolytic clearance, involving cytokine-mediated inhibition of HBV replication without the direct destruction of infected cells. The cells that mediate these early antiviral effects have not yet been identified in human infection. However, studies in chimpanzee and transgenic mouse models have shown that HBV-specific CD8+ T cells (cytotoxic T lymphocytes or CTLs) are important. In the chimpanzee model, monoclonal antibody-mediated depletion of CD8+ T cells at the peak of viremia delays viral clearance until the antibody levels wane and virus-specific T cells return to the liver (128). When HBsAg-specific CD8+ T cells are adoptively transferred to HBV transgenic mice, they recognize their cognate antigen, lyse a small number of local hepatocytes, and, concurrently, produce cytokines that downregulate HBV replication throughout the liver (129–131). HBV-specific CTLs are important effector cells involved in HBV clearance. Intravascular CD8+ effector T cells play a diapedesis-independent immunosurveillance role in the liver, extending cytoplasmic protrusions through endothelial fenestrae to trigger effector function following hepatocellular antigen. This process is inhibited by the sinusoidal defenestration and capillarization characteristic of progressive liver fibrosis, suggesting a mechanism for immune dysregulation in the setting of cirrhosis (132).

The disappearance of most of the HBV DNA from the blood and the liver is followed by the development of clinically apparent hepatitis, with peak alanine transaminase (ALT) levels and maximal CD4+ and CD8+ T-cell responses in the blood (119, 127, 128). Massive chemokine-mediated recruitment of intrahepatic inflammatory cells occurs, including HBV-specific and nonspecific T cells ("bystander lymphocytes"), neutrophils, NK cells, and monocytes/macrophages (126, 133). Much of the hepatocellular damage occurring during acute hepatitis therefore results from secondary, antigen nonspecific, inflammatory responses set in motion by earlier HBV-specific CTL response. Likely, the combination of cytolytic and noncytolytic mechanisms is required to prevent infection of new hepatocytes and clear infected hepatocytes.

Humoral responses are also critical for controlling HBV. HBV clearance is associated with the production of anti-HBs antibodies, and sera with high levels of anti-HBs can control or prevent HBV infection (134). The protective role of antibodies directed against the nonenvelope proteins is not clear; anti-HBc does not confer virus-neutralizing activity. Chimpanzees receiving infusions of anti-HBc and anti-HBe

antibodies both pre- and postinfection with HBV demonstrated markedly prolonged incubation periods not present when anti-HBc alone was infused, suggesting a possible but undefined role for anti-HBe (135).

The integrated activation of both the cellular and humoral arms of the adaptive immune response is therefore required for clearance of acute HBV infection. HBV-specific antibodies, together with HBV-specific memory T cells, then provide protective immunity against future infections.

Noncytolytic Clearance and the Transgenic Mouse Model

Important insights into the molecular mechanisms of noncytolytic clearance have been obtained from the HBV transgenic mouse model (70) (Fig 6). Key roles for the cytokines IFNγ, TNFα, and IFNα/β have been revealed (70). HBV is inhibited by any stimulus that induces these cytokines in the liver, including injection of IL-12 (136), anti-CD40 mAb (137), IL-18 (138), IL-2 (139), α-galactosylceramide (which activates NKT cells) (123), CD4+ T cells (140), other hepatotropic viral (adenovirus, LCMV, MCMV) (141), or parasitic infections (schistosomiasis, malaria) (142, 143). TLR signaling has also inhibits HBV replication in the transgenic mouse model via a type 1 IFN-mediated mechanism (122).

Adoptively transferred HBV-specific CTLs abolish HBV gene expression and replication in the liver of transgenic mice without killing the hepatocytes. This antiviral function is mediated by IFNγ and TNFα secreted either by the CTL or by the antigen-nonspecific macrophages and T cells that they activate following antigen recognition (130). These cytokines activate two independent virocidal pathways. The first pathway eliminates HBV nucleocapsid particles and their cargo of replicating viral genomes by preventing replication-competent capsid assembly (136, 144) in a proteasome- (145) and kinase-dependent manner (146) (Fig 6). The second pathway destabilizes the viral RNA (130) by an SSB/La-dependent mechanism (147–149). The La autoantigen normally binds a predicted stem-loop structure of the 5′ end of the posttranscriptional regulatory element of HBV RNA to protect several endoribonuclease cleavage sites from cellular RNases. IFNγ and TNFα induce proteolytic cleavage of the La autoantigen, thereby allowing destabilization of HBV RNA (147) (Fig 6).

Type 1 IFNs (IFNα/β) also inhibit HBV replication at both pre- and posttranscriptional levels. Transcription of the cccDNA minichromosome is inhibited via epigenetic modification, thus reducing pgRNA levels (150). IFN also activates hepatocellular mechanisms that prevent the formation of replication-competent HBV capsids (144). The molecular mechanism(s) that mediate this inhibition have not yet been defined, but the antiviral activities of double-stranded RNA-dependent protein kinase (PKR) and interferon-regulatory factor 1 (IRF-1) have been implicated (151).

The key role of TNFα was recently highlighted using a murine model of HBV infection with tail vein injection. The cellular inhibitor of apoptosis protein (cIAPs) was shown to attenuate TNFα signaling and restrict the death of infected hepatocytes, allowing viral persistence. Mice with a liver-specific cIAP1- and total cIAP2-deficiency efficiently controlled HBV infection compared with wild type mice. Pharmacological inhibitors of cIAPs were subsequently shown to promote the clearance of HBV infection in the mouse model, emulating the effect of experimentally induced cIAP deficiency (152). Birinapant, a cIAP inhibitor developed for cancer therapeutics, has now entered phase I clinical development.

Chronic Infection

The mechanism of HBV persistence is not fully understood but is likely multifactorial, including strategies of HBV-specific immune suppression and evasion, developmental-dependent and genetic host immunocompetence, and the persistence of stable forms of the HBV genome, such as cccDNA in the form of the minichromosome. Persistent infection is more likely to occur following perinatal transmission or after early horizontal transmission to children or immunocompromised adults. The dose and HBeAg phenotype of viral inoculum may also influence the outcome when a mature immune system is exposed (153).

The most important predictors of persistence appear to be 1) the maturity of the host immune system at the time of infection, as the majority of the world's CHB infections in endemic areas occur between the neonatal period and 5 years of age, and 2) the HBeAg phenotype of the HBV. However, unlike previously thought, the high rate of chronicity is not due to evasion of the immature immune system or induction of immune defects. The dogma that there is an absent or impaired immune response to HBV is at odds with the efficacy of HBV vaccination in infants born to mothers with HBV. The concept of "trained immunity" in infants born to HBV-infected mothers describes a phenomenon in which the functional maturation of neonatal immunity is modulated by extrinsic factors, such as live vaccination, which can subsequently protect newborns against unrelated pathogens by increasing the efficiency of innate immune function (154). One cohort of infants born to HBV-infected mothers, as compared to nonexposed controls, showed enhanced innate immune-cell maturation and Th1 development and *ex vivo* responses to unrelated pathogens (155). Rather than being defective, the immune system of a newborn, it appears, is less likely than that of an adult to trigger a pro-inflammatory reaction, which is possibly an evolutionary adaptation to avoid dangerous immune reactions in the developing fetus (156). Further challenging current "immune tolerant" models, chronically infected adolescents, considered to be in the traditional "immune tolerant" phase, do not exhibit a tolerogenic T-cell profile (157).

Viral Mechanisms of Persistence

All of the major proteins produced by HBV have been implicated in the development of chronicity. The surges in viral load associated with disease flares have been correlated with elevations in IL-10, an immunosuppressive cytokine (158). Elevated IL-10 induces hyporesponsiveness in NK, CTLs, and T helper cells. Additionally, at levels of viremia >7 $\log_{10}$ copies/ml, almost no circulating HBV-specific CTLs can be detected (159).

The continuous production of both HBsAg and HBeAg in concentrations at least 1000-fold higher than that of whole virions is associated with immunological alterations (160). HBeAg is not required for infection or replication, yet it is absolutely required for the development of chronicity. Viral mutants, defective for HBeAg production, may cause acute, even fulminant, hepatitis but not chronic hepatitis B. In murine models HBeAg has a "tolerizing effect" on CTL-mediated clearance of HBcAg-positive hepatocytes (32,161–163), and it likely contributes to the poor core-specific T-cell responses present in patients with HBeAg-positive CHB. HBeAg may generate this tolerance via modulation of various immune components:

- TLR2 expression is downregulated on peripheral monocytes, Kupffer cells and hepatocytes from patients with HBeAg-positive disease but upregulated in HBeAg-negative disease (121). When patient PBMCs are stimulated with the specific TLR2 ligand Pam-2-Cys, reduced TNFα induction is noted in the HBeAg-positive group, confirming functional significance.
- *In vitro* experiments have shown that HBeAg selectively and specifically binds several proteins involved in downstream TLR signaling pathways, thereby downregulating NFκB and IFNβ promoter activity (164, 165). This also suppresses NK cell secretion of IFNγ, normally stimulated by IL-18 (166).
- HBeAg is also associated with downregulation of the costimulatory molecule CD28 on HBV-specific CTLs (167).

The excessive numbers of produced particles that are not infectious may act as a decoy for HBV-specific humoral immunity and also promote a state of T-cell anergy and deletion. In murine and cell line experiments, HBsAg may directly modulate innate immune-signaling pathways, namely NFκB and MAPK, suppressing inflammatory cytokines and interferon gene transcription normally upregulated by TLR signaling (165,168–170).

The HBx protein may contribute to establishing persistent infection by inhibiting proteasome-based degradation of viral protein to reduce HBV antigen presentation (70). Additionally, *in vitro* HBx binds the β-interferon-promoter stimulator-1 (IPS-1), enhancing its degradation (171), and also disrupting signaling pathways responsible for IFNβ gene transcription (172, 173). HBx has also been associated with alterations in the balance of pro- and anti-inflammatory cytokines, but the biologic significance of these alterations is yet to be defined in human infection (174, 175).

The HBV Pol protein suppresses the production of the MyD88 adaptor protein, which is central to TLR function (176), by downregulation of the STAT-1 nuclear transcription factor via 1) competitive binding of importin-α5 (the nuclear entry transporter for STAT-1) by the RNaseH domain (177), and 2) inhibition of protein kinase C-δ, responsible for STAT-1 phosphorylation, by the TP-domain (177, 178). The biologic effect of this pathway has been demonstrated in murine models (179). Pol also blocks IRF-3 phosphorylation, which is part of the TLR-3 signaling pathway to produce IFNβ (173, 180).

Selection of mutations in the HBcAg by immune pressure may result in T-cell anergy. Analysis of HBcAg epitopes in chronically infected patients identified novel mutations that, when presented, resulted in antagonism of the T-cell receptor and functional inactivation (181). These mutations were HLA-A2 restricted and located in the major HBcAg epitope between residues 21 and 27. The significance of these mutants in a larger population has not been determined.

Immune Defects in Chronic Infection

The immune dysfunction present in individuals with CHB manifests in the effector cells of both the innate and adaptive arms and may occur as a consequence of chronic immune stimulation. HBV-specific CD4+ and CD8+ T-cell responses are significantly diminished relative to individuals with resolved infection. The frequency and function of both intrahepatic and peripheral HBV-specific T cells are inversely proportional to the level of circulating HBV DNA in CHB (159), and, in animal models, the sustained presence of viral antigens may lead to progressive anergy and ultimately deletion of T cells (182, 183). The mechanisms that may contribute to this are discussed below.

Molecular Markers of Exhaustion

Exhaustion of immune effector cells is a known consequence of chronic viral infections; upregulation of molecules responsible for suppressing CTL function has been observed in both animal models and patients with CHB. The programmed death-1 receptor (PD-1) is overexpressed on HBV-specific CTLs in patients with CHB (184–186) with a corresponding increased expression of the PD-1 ligand on the surface of dendritic cells (DCs) in response to IFNα and IFNγ (187). Activation of PD-1 by its ligand increases the sensitivity of a cell to apoptosis. Inhibition of PD-1 expression and signaling is now being evaluated as a possible therapeutic strategy to reverse the immune exhaustion associated with CHB.

T-cell immunoglobulin domain and mucin domain-containing molecule-3 (Tim-3) is also upregulated on the surface of both CD4+ and CD8+ T cells in CHB patients (188). Tim-3 activates cellular pathways responsible for functional inactivation and apoptosis. Consequently, Tim-3 upregulation results in impaired cellular proliferation and cytokine production (189). *In vitro* blockade of Tim-3 can restore cellular immune function (190). The natural ligand for Tim-3 has been shown to be galectin-9, levels of which are elevated in CHB patients (189). The source of galectin-9 has not been determined.

The exhaustion profile of both CD4+ and CD8+ HBV-specific T cells also includes overexpression of the NK cell receptor 2B4 (NK2B4) (191) and Bcl-2-interacting mediator (Bim) (192), which are involved in cytokine production and apoptosis, respectively. The inhibitory costimulatory molecule CTLA4 is also upregulated on CD8+ T cells (193), and the positive costimulatory ligand of CD40 is downregulated on CD4+ T cells (194). These changes result in suppression of signaling molecules associated with CD4+ cellular proliferation (195) and increased production of IL-10 (194).

Innate Immune Dysfunction

The innate immune system is implicated in the pathogenesis of CHB. Type 1/3 IFN induction is triggered by TLR signaling in response to viral pathogen-associated molecular patterns (PAMPs), and TLR-7 ligands have now entered clinical development for HBV (see below). Dysregulation of TLR signaling contributes to immune evasion in CHB and impaired expression of TLR-3 and 9, as well as of TLR-2, and downstream signaling pathways have been described (196–198). More recently, miRNAs have been proposed to negatively regulate TLR signaling in the setting of CHB, specifically targeting TRAF6 and other key effectors of TLR signaling, including STAT-1 (199, 200). miR-146a is overexpressed by both CD4+ and CD8+ T cells in CHB patients, and *ex vivo* experiments reveal its overproduction in response to PBMC stimulation by HBV antigens, TNFα, and IFNα (200). Blockade of miR-146a *in vitro* restored HBV-specific CTL responses. miRNA may also directly regulate HBV replication and has been implicated in the pathogenesis of HBV-related HCC.

Regulatory T Cells

The T-cell subset characterized by CD4+CD25+FoxP3+ expression, known as regulatory T cells, or Tregs, are known to modulate the immune system to maintain tolerance to self-antigens and to abrogate autoimmune disease. Some pathogens can manipulate Tregs to selectively immunosuppress

host immunity and promote persistence. Intrahepatic levels of Tregs are elevated in patients with chronic HBV (186,201–203), and their frequency correlates with both viral load and inflammatory activity (201, 204). *Ex vivo* experiments demonstrate the suppressive effect of Tregs on HBV-specific CD4+ and CD8+ T cells from patients with CHB (201, 205). Tregs may contribute to a proviral intrahepatic cytokine environment. Intrahepatic Tregs are increased in woodchucks chronically infected with WHV and have been associated with IL-12 nonresponse (202).

Adaptive Immune Dysfunction

Chronic hepatitis B is characterized by a failure to mount an efficient adaptive immune response. In addition to cellular exhaustion detailed above, γδ-T cells (γδTC), have been implicated in an immunosuppressive role in CHB. *In vitro* these cells from CHB patients can dampen the production of inflammatory cytokines by inhibiting their production from Th17 cells and CTLs (206, 207). The frequency of γδTC production is increased in immune-tolerant patients (207). These cells also liberate cytokines that recruit intrahepatic myeloid-derived suppressor cells, which also lead to CTL suppression (208). In addition, the dying hepatocyte releases large quantities of arginase into the circulation. Arginase enzymatically degrades the amino acid l-arginine, which is essential for the function of the intracellular signaling zeta (ζ) domain of the T-cell receptor (TCR). The CTLs of CHB patients downregulate TCRζ, with the functional consequence of impaired IL-2 secretion (209). Overall this results in reduced antiviral function (210).

Humoral Immunity

B cells and humoral immunity play a critical role in CHB. HBV-vaccinated individuals have anti-HBs antibodies that are 95% protective against initial infection (211). Treatment with specific anti-B cell therapy (rituximab, ofatumumab) confers a 30 to 60% risk of viral reactivation. This risk persists even in those patients who have achieved HBsAg seroconversion and is much higher than if patients were treated with traditional immunosuppressive agents (212–215). Additionally, a new subset of B cells, called regulatory B cells, is characterized by the ability to secrete the potent immunosuppressive cytokine IL-10 (216). The full functions of this B cell subset, which is rare in the peripheral circulation, are yet to be elucidated, but their frequency increases in proportion to disease flares in CHB. This results in increased IL-10 secretion and subsequent CTL suppression (217).

Host Genomic Factors

Genetic predisposition to the development of chronic HBV infection has not been studied extensively. A genome-wide association study in Japanese patients identified 11 single nucleotide polymorphisms associated with CHB risk that were localized to the HLA-DP locus, thus implicating MHC class II antigen presentation as a possible factor (218). Reinforcing the role of the HLA-DP locus, a separate study identified other polymorphisms that were associated with an increased chance of spontaneous HBsAg clearance at a younger age (219).

IL-28B genotyping, which is predictive of interferon-based treatment responses in chronic hepatitis C infection, may also influence the development of chronic hepatitis B infection, as well as the response to both IFN- and lamivudine (LMV)-based therapy (219–223). IL28B genotyping has been linked to rates of HBsAg loss following long-term IFN-α therapy (224).

CLINICAL MANIFESTATIONS

Acute Hepatitis B

The incubation period of acute hepatitis B varies from 1 to 4 months postinfection. Clinical presentation varies from asymptomatic infection in two-thirds of patients to frank icteric hepatitis and, rarely, fulminant liver failure. A serum sickness-like illness, characterized by fever, arthralgias, and rash, may develop in the prodromal period, followed by constitutional symptoms, anorexia, nausea, jaundice, and right upper-quadrant discomfort. Clinical symptoms coincide with the biochemical abnormalities that develop on liver function testing. The biochemical diagnosis of acute hepatitis is characterized by elevations in the concentration of serum ALT and bilirubin. ALT values in AH-B are usually greater than 500IU/ml, with ALT being higher than AST. Elevations in bilirubin are usually modest, 5 to 10 mg/dl (85 to 170 umol/l). The symptoms and jaundice generally disappear after 1 to 3 months, although fatigue may persist in some patients even after normalization of liver function tests.

Fulminant Hepatic Failure

The most serious complication of HBV infection is fulminant hepatic failure (FHF). This is unusual, occurring in $<0.5\%$ of patients and is defined as the onset of hepatic encephalopathy within 8 weeks of the development of jaundice. The risk of FHF may be higher in patients acutely infected with basal core promoter or precore variants (225), coinfected with other hepatitis viruses, or with underlying liver disease. The development of coagulopathy (marked by an international normalized ratio [INR] >1.6) should raise concern about the risk of development of FHF and warrants consultation with a liver transplant center as the prognosis is poor, carrying an 80% mortality rate, if left untreated. Prompt institution of nucleos(t)ide therapy to reduce viral load has reduced the mortality rate to 20% in some series (226).

Chronic Hepatitis B

Most cases of CHB occur in endemic populations as the result of perinatal or early childhood horizontal transmission. The natural course of disease is determined by the interplay between virus replication and the host immune response and may be divided into four phases: 1) the immune tolerance phase; 2) the immune elimination phase; 3) the immune control phase; 4) HBeAg-negative CHB or immune escape phase (Fig 7).

The immune-tolerance phase is characterized by high levels of viral replication, with HBV viral load up to 10^{12} IU/ml and HBeAg detectable, but no evidence of active liver disease as manifested by lack of symptoms, normal serum ALT measurement, and minimal changes on liver biopsy. HBV-specific T-cell responses are weak or undetectable in this phase (159, 227). The immune-tolerant phase usually lasts 10 to 30 years, during which time there is a very low rate of spontaneous HBeAg clearance, reported to be only 15% after 20 years of infection (228, 229).

Transition from the immune-tolerance to the immune-elimination phase typically occurs during the second and third decades of life. This transition is marked by increased HBV-specific T-cell immunity, falling HBV-DNA titers, increased serum ALT, and necro-inflammatory histological changes. Immune pressure drives an increase in viral quasi-species diversity. The immune-elimination phase may last for years, during which time disease activity fluctuates and progressive liver damage accumulates. The most important factor influencing prognosis appears to be prolonged viral

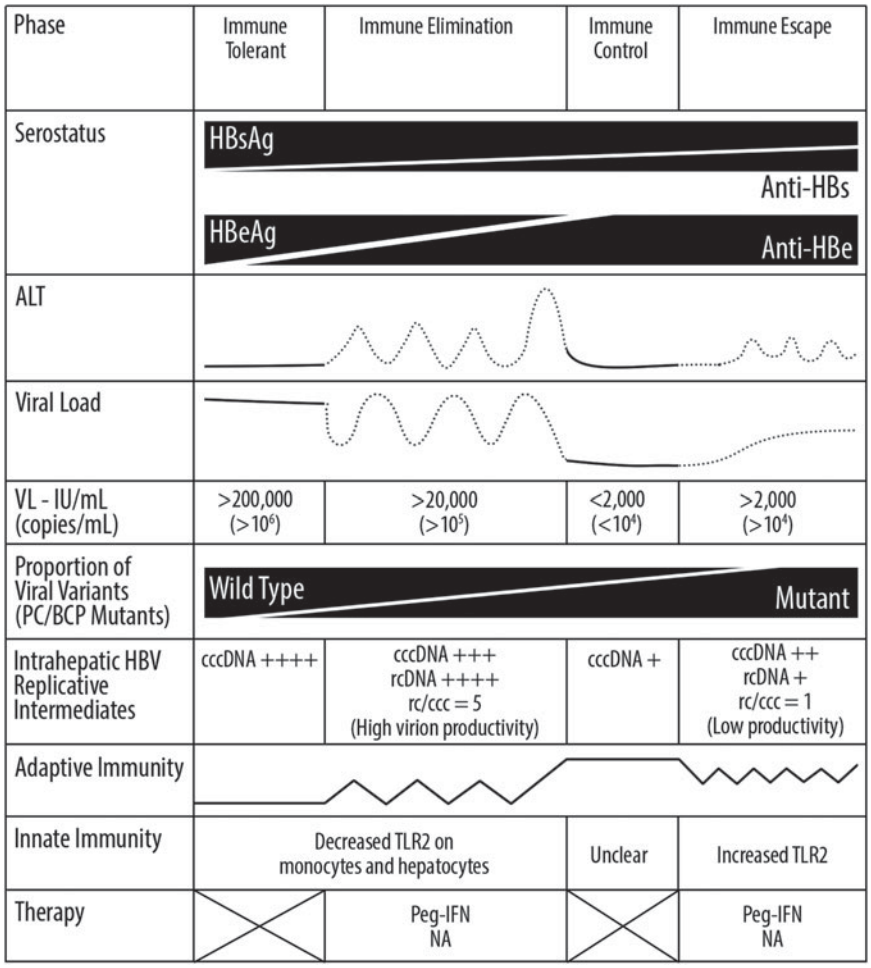


FIGURE 7 The natural history of chronic hepatitis B, showing relationships between serology, biochemistry, molecular virology (serum and liver compartment), as well as immunological parameters of the innate and adaptive arms.

replication at levels >2000 IU/ml. In a small percentage of patients, severe ALT flares result in hepatic decompensation and, rarely, death from hepatic failure. Spontaneous HBeAg seroconversion, the serological marker of the end of the immune-elimination phase, increases to an annual rate of 10 to 20 percent during the immune-elimination phase (228, 229). HBeAg seroconversion correlates with a significant drop in viral load, often to undetectable levels, and histological arrest or improvement. HBV is not cleared from the liver, however, and the nuclear cccDNA reservoir persists.

Patients in the low or nonreplicative phase are HBeAg-negative and anti-HBe positive. A serum HBV-DNA threshold, below which HBV replication is not thought to be clinically significant, has been set at 2,000 IU/ml (230). In some patients, serum HBV DNA is no longer detectable. Liver disease remits as evidenced by normal serum ALT concentrations and resolution of necroinflammation in liver biopsies. HBV-specific T-cell reactivity is high and thought to maintain viral suppression (231). The remaining HBV population is genetically diverse, with positive selection of viral variants defective for HBeAg production that are able to persist but at the cost of a reduced replication phenotype (232, 233). The annual rate of delayed clearance of HBsAg has been estimated to be 0.5 to 2% in Western patients and much lower (0.1 to 0.8%) in Asian countries (234, 235). HBsAg seroconversion is regarded as a functional "cure" and is associated with a good prognosis. However, it is unlikely that HBV DNA is ever completely cleared from the liver, and a small risk of reactivation persists in the setting of potent immune suppression (e.g., allogeneic bone marrow transplantation or B-cell depletion using therapeutic monoclonal antibodies).

A percentage of patients continue to have moderate levels of HBV replication and active liver disease (elevated serum ALT and chronic inflammation on liver biopsies) but remain HBeAg-negative. This typically occurs following the emergence of variants of HBV that are phenotypically HBeAg-negative and genotypically include PC or BCP mutants. It remains unclear whether this occurs as a smooth transition from the HBeAg-positive immune-elimination phase or as reactivation from the low-replicative phase.

In adult-acquired CHB, the immune-tolerance phase is typically absent, with disease progressing immediately to the immune-elimination phase. Some patients will be immunosuppressed, and, in this setting, the disease course may be more aggressive. HIV coinfection is associated with an increased risk of cirrhosis and liver-related morbidity (236).

CHB affects nearly 10% of HIV-infected patients. HIV directly impacts the outcome of HBV infection, complicating its natural history, diagnosis, and management. In the

setting of HIV and HBV coinfection, levels of serum HBV DNA tend to be higher, and spontaneous HBeAg seroconversion occurs at a lower rate. For reasons that remain unclear, liver damage, especially fibrosis, progresses at a higher rate than in HBV monoinfection, despite hepatic necroinflammation typically being less severe. With improved control of HIV disease with highly active antiretroviral therapy (HAART), liver disease has emerged as one of the leading causes of death in patients with HIV (236). Therefore, all patients with HIV should be screened for HBV infection. It is recommended that testing for both anti-HBc and HBsAg be performed, as patients with HIV can have occult HBV. Similarly, all HBV patients should undergo HIV testing (230).

Complications of Chronic Hepatitis B

The major complications of CHB are cirrhosis, hepatic decompensation, and HCC. Between 15 and 40% of individuals with CHB will develop at least one of these sequelae during their lifetime, with the highest risk in men (237, 238). CHB has a variable natural history, and accurately predicting prognosis in an individual patient is difficult. A key role has, however, been identified for viral load 10^4 copies/ml (>2,000 IU/ml), and age greater than 30 years, as risk factors for clinical progression to cirrhosis and HCC (3, 4).

Cirrhosis

The current challenge in CHB is to identify patients at risk for progressive liver disease so that therapy may be offered early to alter the natural history. The major risk factors for progression to cirrhosis are viral load, the presence of fibrosis on liver biopsy, and elevated serum ALT. Other factors that influence progression to cirrhosis include viral genotype, HBeAg positive disease, level of HBsAg, coinfection with other viruses, such as hepatitis D virus (HDV) and HIV, and alcohol consumption.

In patients with CHB, the strongest independent predictor of progression to cirrhosis is HBV viral load. In a prospective study of 3582 community-based HBsAg-positive Taiwanese nationals, followed for a mean of 11 years, the risk of cirrhosis increased significantly as baseline viral load increased (3). A critical-threshold viral load of 10^4 copies/ml (2,000 IU/ml) was identified (Table 2 and Fig 8A). The effect of viral load was independent of age, sex, cigarette smoking, alcohol consumption, HBeAg status, and alanine transaminase level. On multivariate analysis male sex, older age, HBeAg-positivity, and 45 IU/ml ALT at the time of study entry were also identified as independent predictors for cirrhosis. The annual incidence of cirrhosis was 0.9%, consistent with the 0.7% reported by a previous study of asymptomatic HBV carriers (239), but lower than the reported rate of 2 to 7% observed in tertiary referral-center populations (240–242). It is likely that the risk associated with absolute viral load at entry reflects duration of viremia and older age. High viral load in younger patients may not have the same prognostic implications, particularly for those still in the immunotolerant phase of disease. Whether the same risk applies to HBsAg carriers in Western countries with adult-acquired HBV infection also remains unclear.

A second prospective study conducted in mainland China has confirmed these data (243). A high baseline viral load (10^5 copies/ml or 20,000 IU/ml) predicted severe liver disease and HCC 11 years later, in comparison to HBsAg-positive individuals with a baseline undetectable viral load. Furthermore, high viral load at baseline predicted liver-associated mortality. Mortality was substantial, with more than 20% of individuals with a high viral load at baseline dying of chronic liver disease or hepatocellular carcinoma by the end of follow-up. A more detailed stratification of viral load in this study was limited by sample size.

TABLE 2 Risk scores assigned to predictors of liver cirrhosis and HCC determined from a large, longitudinal Asian cohort of CHB patients, used in the developed risk nomograms shown in Figure 8

Baseline Predictor	Risk Score for Liver Cirrhosis	Risk Score for HCC
Age (each 5 years increment)	1	1
Sex		
Female	0	0
Male	4	2
Levels of ALT (IU/l)		
<15	0	0
15–44	1	1
≥45	3	2
HBeAg | HBV DNA | HBsAg | Genotype		
Negative | $<10^4$ | <100 | Any type	0	0
Negative | $<10^4$ | 100–999 | Any type	3	2
Negative | $<10^4$ | ≥1000 | Any type	4	2
Negative | 10^4 – 10^6 | <100 | Any type	5	3
Negative | 10^4 – 10^6 | 100–999 | Any type	5	3
Negative | 10^4 – 10^6 | ≥1000 | Any type	7	4
Negative | ≥10^6 | Any level | B or B + C	7	5
Negative | ≥10^6 | Any level | C	13	7
Positive | Any level | Any level | B or B + C	7	6
Positive | Any level | Any level | C	10	7
Family History of Hepatocellular Carcinoma		
No	-	0
Yes	-	2

(Adapted with permission from Lee et al. [248]).

Although elevated serum ALT, especially frequent ALT flares, is a risk factor for progressive disease (241, 242, 244, 245), mild elevations of serum ALT are not necessarily reassuring. Hong Kong patients with serum ALT in the range of 1 to 2 times the upper limit of normal (ULN) have a greater risk of clinical complications than patients with serum ALT 2 times the ULN (246). Furthermore, serum ALT 0.5 to 1 times the ULN was associated with a greater risk of complications than a low-normal serum ALT (<0.5xULN). Hence, patients with minimal enzyme elevations may have progressive disease. Studies that have considered ALT over time have suggested that progression of fibrosis in patients with normal ALT may be limited to those patients who subsequently develop ALT elevation (247).

Measurement of quantitative HBsAg levels is becoming more widespread and useful in cirrhosis risk prediction (248). Hazard ratios of 1.96 (CI: 1.32–2.90) and 3.60 (CI: 2.54–5.10) were found for HBsAg levels of 100 to 999 IU/ml, and ≥1000 IU/ml, respectively, in progression to cirrhosis.

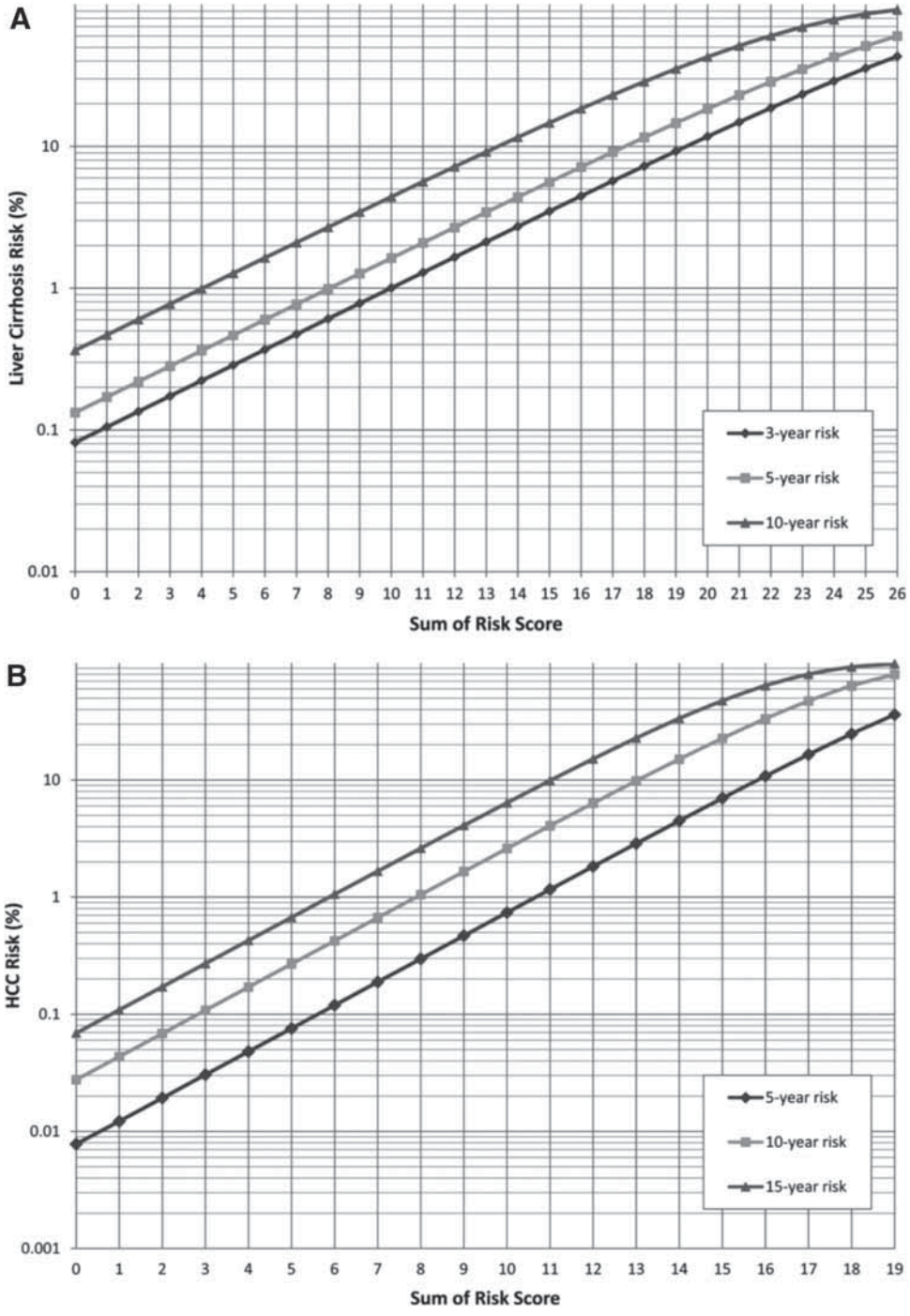


FIGURE 8 Nomograms for the predicted risk of (A) liver cirrhosis (low risk <11; moderate risk 11 to 16; high risk ≥17), and (B) HCC (low risk <9; moderate risk 9 to 12; high risk ≥13). These have been developed using identified virologic and host factors that increase risk of complications. The scoring system is outlined in Table 2. Reproduced with permission from Lee et al. (248).

Liver biopsy remains a useful clinical tool with prognostic significance. The probability of evolution to cirrhosis is significantly higher in patients with moderate-to-severe inflammatory changes, particularly if bridging hepatic necrosis is present (241, 242,249–252). Advanced fibrosis (Scheuer score 2/3) is also a risk factor for the development of cirrhosis (244). The combination of aggressive inflammation and fibrosis has also been linked to survival (253). Liver histology can also worsen rapidly in patients who have recurrent exacerbations or reactivations of hepatitis (230).

Viral factors, including both HBV genotype as well as common HBV variants, have been shown to influence the risk of disease progression. In Asian countries, where genotypes B and C predominate, more rapid and frequent progression to cirrhosis has been noted in patients with genotype-C disease (254–258). Much of this risk is

explained by the observation that genotype-C patients display more active hepatitis, and a later age of seroconversion, compared to genotype-B patients, confirming the critical role of prolonged viremia and inflammation in the development of clinical complications. Genotype A and D are more common in Western populations. Genotype-D infection has been associated with more advanced disease (259). There are no natural history studies that compare all 4 of the major genotypes. In one Japanese study, genotype A was associated with milder disease than genotypes B and C (260).

Among common viral mutants, the BCP mutation appears to be a risk factor for cirrhosis (261–265). The role of the PC variant, the most common variant associated with HBeAg-negative disease, remains unclear. Loss of HBeAg and emergence of the PC variant are now recognized to occur with increasing duration of disease, typically in the third or fourth decade. In treatment-naïve patients in France, advanced fibrosis tended to be higher in patients with PC mutant-positive and BCP mutant-negative infection (266). However, the advanced disease seen in patients in these selected cross-sectional studies may simply reflect persistent, long-standing viremia rather than a pathogenic role for precore mutants. Both genotype and BCP variants have also been linked to the development of HCC.

Several large Asian studies have demonstrated an increased risk of cirrhosis in CHB patients with comorbid metabolic syndrome or diabetes mellitus. One prospective study of 1466 CHB patients characterized their metabolic risk profile and liver fibrosis stage by transient elastography (TE), with a 10% subset undergoing formal liver biopsy, demonstrating good correlation between TE and histology. The presence of features of metabolic syndrome, including type II diabetes mellitus, hypertension, dyslipidemia, and central obesity, was associated with a higher risk of cirrhosis on multivariate analysis, independent of the traditional risk factors outlined above (267). A longitudinal follow-up study of this cohort identified an increased risk for liver fibrosis progression in patients with metabolic syndrome (odds ratio of 2.0 [CI: 1.1–3.5] for fibrosis progression, defined as an increase in liver transient elastography measurement of ≥30% [268]).

Other risk factors associated with increased rates of cirrhosis include habitual alcohol consumption and concurrent infection with HCV, HDV, or HIV (269, 270).

Although, intuitively, the conclusion that long-term viral suppression will prevent the development of cirrhosis remains unproven. However, two prospective studies of CHB patients achieving several years of sustained virologic suppression using either entecavir (ETV) or tenofovir have demonstrated regression of advanced fibrosis or cirrhosis (271, 272). Additionally, studies of the older antiviral drugs have shown improvement in histologic fibrosis scores (273).

Hepatic Decompensation

Decompensated liver disease develops as a complication of cirrhosis. The estimated 5-year risk of progression in CHB to decompensation is 20 to 23% (274). Risk factors for decompensation include persistent viremia, age, and markers of impaired synthetic function (including low albumin, low platelets, high bilirubin, and ascites) (275–277). Once decompensation occurs, the estimated 5-year survival is 14 to 35%, compared to 80 to 85% for compensated patients. The advent of antiviral therapy has had a dramatic impact on this natural history, again emphasizing the critical role viral replication plays in disease progression.

Hepatocellular Carcinoma

Seropositivity for HBsAg is one of the most important risk factors for the development of HCC. The relative risk of developing HCC, if HBsAg-positive, has been found to vary from 7-fold in Japan to 60- to 98-fold in Taiwan, likely varying with the population attributable risks of 10% and 94%, respectively (278, 279). More important for clinical practice is population stratification for individual patients with CHB.

HBV viral load has a profound impact on the development of HCC (Table 2 and Fig 8B), and a biological gradient of risk has been seen according to HBV viral load (4). The cumulative incidence of HCC over 13 years of follow-up was 1.3 versus 14.9 percent for baseline HBV DNA levels of <300 and $>10^6$ copies/ml (<60 and $>200{,}000$ IU/ml), respectively. The impact of persistent viremia was assessed by measuring viral load at last follow-up (the median time between samples was 10 years). Individuals were at highest risk if the viral load was $>10^5$ copies/ml ($>20{,}000$ IU/ml) both at baseline and follow-up. Conversely, a significant reduction in HCC risk was observed in those who had a high entry viral load at baseline, but in whom viral load was significantly reduced at follow-up. Of known covariates, cirrhosis at baseline, male sex, older age, alcohol consumption, and HBeAg-positivity were identified as independent risk factors. The presence of baseline cirrhosis was associated with the highest hazard ratio (HR) for development of HCC (HR 9.1, 95% CI 5.9—13.9).

The importance of viral load in hepatic carcinogenesis is further emphasized by the findings of an association between effective viral suppression using NA and reduced risk of HCC over time. Importantly, all studies have shown that the HCC risk is not completely eliminated, especially in cirrhosis, and lifelong surveillance for HCC continues to be recommended. It should be noted that the most robust data are available only for Asian populations. There are no comparative studies in Caucasian or African patients.

The risk of HCC has been independently associated with HBsAg levels in the Risk Evaluation of Viral Load Elevation and Associated Liver Disease/Cancer-Hepatitis B Virus (REVEAL) cohort, particularly at levels >1000 IU/ml (248). A separate study, also performed in a large population from Taiwan, demonstrated a 13-fold increase in risk associated with HBsAg >1000 IU/ml in HBeAg-negative patients classified as inactive carriers (i.e., HBV viral load <2000 IU/ml) (5). This suggests a clinical use for quantitative HBsAg measurement with regards to HCC risk stratification. The applicability of these results to Western populations is again uncertain.

Asian studies have identified viral factors that are associated with the development of HCC. Genotype C is associated with increased risk compared to genotype B (248, 280, 281) and genotypes A and D (282). Mutations in the viral genome have also been linked to hepatic carcinogenesis, in particular those located in the BCP and Pre-S regions (283). Of these, BCP mutations are the most strongly associated, conveying an almost 4-fold increase in HCC risk compared to wild type virus (17, 283). Interestingly, no increase in risk has been associated with precore mutant virus (283).

Extrahepatic Manifestations

Chronic HBV infection is associated with several extrahepatic manifestations that are thought to be mediated by circulating immune complexes. These include the serum sickness-like prodrome of acute HBV infection, polyarteritis nodosa, HBV-associated glomerulonephritis, mixed essential cryoglobulinemia, and neurological manifestations (284).

A serum sickness-like prodrome precedes clinical hepatitis by 1 to 6 weeks in 10 to 30% of those acquiring acute HBV infection. Also known as the "arthritis-dermatitis" syndrome, it is characterized by a symmetrical generalized inflammatory arthritis, typically involving the small joints of the hands and feet, which may be indistinguishable from acute rheumatoid arthritis. The joint lesions are nondestructive, however. Skin manifestations are variable, occurring in more than 50% at the time of, or shortly after, the joint symptoms. Lesions described include maculopapular, petechial or purpuric rash, palpable purpura, Henoch-Schönlein-type purpura, erythema multiforme, toxic erythema, lichenoid dermatitis, and urticaria. Fever is common. Renal involvement with proteinuria or hematuria is much less common. Angioneurotic edema may rarely occur.

Polyarteritis nodosa (PAN) is a rare but serious complication of chronic HBV infection. The syndrome normally presents within 4 months of the clinical onset of HBV infection, with abdominal pain due to arteritis of medium-sized vessels causing ischemia of the intestine and gallbladder. The finding of microaneurysms of blood vessels in the renal, hepatic, or mesenteric circulations on angiography is virtually pathognomonic. Tissue biopsy of affected organs revealing inflammation of the medium-sized arteries confirms the diagnosis. Treatment involves antiviral therapy, immunosuppression, and plasma exchange. The prognosis is poor without treatment, with a mortality of up to 50%.

Glomerulonephritis (GN) is more commonly associated with CHB. The most common presentation is nephrotic syndrome. A number of patterns of glomerular injury have been described, including membranoproliferative GN, membranous GN, and rarely mesangial proliferative GN. In children the disease is usually self-limited; however, in adults, progression to renal failure has been described.

Other manifestations of HBV-related vasculitis include mononeuritis multiplex and polyneuropathy. Guillain-Barré syndrome has been reported. Associations with polymyalgia rheumatica, polymyositis, and HLA-B27-positive ankylosing spondylitis have also been reported, although a causative link remains controversial.

Occult HBV Infection (OBI)

Of patients who have achieved HBsAg loss or seroconversion, following either acute or chronic HBV infection, an unknown proportion will have detectable HBV DNA in the liver. In this situation, viral DNA may or may not be detectable in the serum (285). When detectable, the serum viral load is low (often < 200 IU/ml). This distinguishes true OBI from "false OBI" where the viral load is high but HBsAg is negative, an uncommon situation that is due to viral variants that produce an antigenically modified HBV S protein not detectable using commercial HBsAg assays, or carry mutations that inhibit S gene expression (286). Occult expression results from strong immune control of viral gene expression (287), and the molecular basis of OBI is related to long-lasting persistence of cccDNA within hepatocyte nuclei (288). The epidemiology of occult HBV remains poorly defined, but it has a worldwide incidence and appears to be more common in endemic areas, in the setting of coinfection with HCV and HIV, in patients with a history of injecting drug use, in hemophiliacs, and in patients on hemodialysis (286). It remains rare in developed countries.

Occult HBV carriers may be a source of HBV transmission in the case of blood donation (289), although the incidence of this has been reduced significantly due to the widespread adoption of nucleic acid testing of donations rather than reliance on serologic markers (288). There is a risk of viral reactivation in OBI patients who are immunosuppressed (290, 291). OBI may be linked with the development of HCC. In one study of a cohort of 69 HCC patients, 75.5% of tumors from the OBI group (HBsAg negative, but HBV DNA detected in tumor tissue) had HBV DNA integrated into host chromosomes (292). This was comparable to the 80% of HBsAg positive groups and in contrast to 0% of the controls. A similar association was found in patients classified as having a cryptogenic cause of HCC (293). OBI might also be a cofactor in the development of HCV-related HCC (294).

LABORATORY DIAGNOSIS

HBV infection is usually diagnosed by serological assays to detect specific antigens (HBsAg, HBcAg, and HBeAg) and corresponding antibodies (anti-HBs, anti-HBc, and anti-HBe). The assays for serum HBsAg and its resultant antibody are critically important because the persistence of HBsAg for longer than 6 months defines chronic infection, and the presence of anti-HBs indicates disease resolution or immunity after vaccination. Beyond diagnosis, novel quantitative assays for HBeAg and HBsAg and ultrasensitive assays for anti-HBe and anti-HBs are providing new insights into the dynamics of humoral immunity in the setting of CHB. The quantification of HBV DNA has recently become a primary tool in the management of CHB. Data are emerging on the relationship between the magnitude of HBV DNA load and the relative risk of developing liver disease (3, 4). Consequently, HBV DNA now plays a central role in defining antiviral treatment eligibility and efficacy. The development of resistance can also be identified by an increase in the level of HBV DNA, generally considered to be a greater than one $\log_{10}$ increase from nadir in an adherent patient (295). Recent advances in molecular diagnostics allow analysis of intrahepatic HBV replicative intermediates, such that comparison of the peripheral serum compartment to the liver compartment is possible.

Serological Markers of Infection

Acute Hepatitis B

Of the serological markers associated with acute infection (Table 3), HBsAg is typically the first to become detectable (296). It appears early, usually some 6 to 12 weeks after exposure, and is present before the onset of symptoms. HBeAg appears soon after HBsAg and is a useful marker of replication activity. As the antigen titers peak, the serum alanine aminotransferase (ALT) levels begin to rise and symptoms first become apparent. Antibodies to the antigens begin to appear during this symptomatic phase. The first antibody raised is IgM directed against the core antigen, anti-HBc IgM (296). In combination with the presence of HBsAg, anti-HBc IgM is the best indicator of acute infection. Anti-HBc IgM peaks in early convalescence before gradually declining over 3 to 12 months, irrespective of whether or not the disease becomes self-limiting or progresses to chronic disease. As the HBc IgM titer falls, a corresponding increase in titer of anti-HBc IgG occurs, which typically remains detectable for life (296). Anti-HBe is either detected concurrently with, or soon after, the appearance of anti-HBc IgM, and its appearance is associated with the rapid clearance of HBeAg. The seroconversion of HBeAg to anti-HBe coincides with a dramatic increase in ALT, reflecting the increased immune-mediated lysis of

TABLE 3 Profile of the serological markers for hepatitis B virus infection

	Incubation Period	Acute Hepatitis	Recovery Period	Immunity	Vaccine Immunity	Chronic Infection	Occult Infection
HBsAg	±	+	±	-	-	+	-
Anti-HBs	-	-	-	+	+	-	±
HBeAg	±	-	-	-	-	±	-
Anti-HBe	-	+	+	+	-	±	±
Anti-HBc IgM	-	±	-	-	-	-	-
Anti-HBc total	-	±	+	+	-	+	±

infected hepatocytes (297). Often anti-HBe will persist for years, but in the absence of any active viral replication, the titer declines. Finally, anti-HBs appears, although it may not be detectable for 3 to 6 months. Anti-HBs is the neutralizing antibody and a marker of disease resolution. In some patients, there may be a "window period" when HBsAg cannot be detected and anti-HBs has not yet appeared; however, anti-HBc IgM is usually found at this time. Anti-HBs is also the marker for confirming successful vaccination, should be the only hepatitis B antibody found (Table 3), and provides protective immunity.

There is no major role for the detection of HBV DNA for the diagnosis of acute infection, as the "window period" is typically asymptomatic and patients will not present for clinical testing. The use of DNA amplification techniques, such as the polymerase chain reaction (PCR), allows detection of HBV DNA in the serum a few weeks after infection, but there is little advantage over the newer, more sensitive HBsAg detection assays (70, 298, 299).

Chronic Hepatitis B

The persistence of high levels of HBsAg for >6 months following acute hepatitis indicates the development of chronic infection (Table 3) (211). The serological profile correlates with acute infection, but as the levels of ALT and anti-HBc IgM decline, HBsAg remains. In the early stages of chronic infection, ongoing replication is indicated by the presence of HBsAg, HBeAg, and HBV DNA. The antibody response consists mostly of anti-HBc IgG, measured as total anti-HBc, with minimal contribution from anti-HBc IgM. HBeAg titers may decline over time, with eventual seroconversion of HBeAg to anti-HBe. High levels of secreted HBe protein are found in the so-called tolerant phase of chronic infection and are associated with very high viral loads and near-normal liver histology. Hepatitic flares, produced as a consequence of heightened immune reactivity to virus-infected hepatocytes, usually accompany elimination of HBeAg. The viral load drops significantly during these flares, presumably as a consequence of the antiviral activity of the host's immune response.

This HBeAg seroconversion occurs at a rate of 5 to 20% per year in adults, and the flare in disease activity at this time is known as the seroconversion illness. A number of abortive flares of active hepatitis may occur over several years before final anti-HBe seroconversion. In most cases, the loss of HBeAg is associated with a decrease in circulating levels of HBV DNA (which may become undetectable), normalization of ALT levels, and a significant clinical improvement despite the lasting presence of HBsAg. It is increasingly recognized, however, that HBV DNA may remain detectable, and disease progresses as HBeAg-negative CHB.

Viral Load Assays

HBV DNA detection and quantification is a key prognostic marker and, as such, provides an important parameter for determining eligibility for antiviral therapy. Serial viral load measurements are now the standard of care for monitoring patients on therapy, both for efficacy and the development of drug resistance. Additionally, mathematical modeling of the kinetics of the decline in viral load has allowed the efficacy of antiviral drugs to be compared and the potential additive or synergistic effects of drug combinations to be examined (300, 301). Current commercial assays utilize real-time PCR, with wider dynamic ranges and lower limits of detection, that overcome problems with quality control, lack of standardization, and limitations in sensitivity of prior assays.

Quantitative HBsAg Assays

First described in 2004, a number of commercially available ELISA assays are available for quantitative HBsAg titer (302–307). HBsAg assays detect virion-associated envelope protein as well as subviral particles. HBsAg levels may therefore reflect hepatocyte cccDNA level and cccDNA transcriptional activity, as well as integrated HBV sequence meaning that the clinical utility of measuring HBsAg levels may relate to a semi-quantitative measure of overall liver "HBV load" (308). During the natural history of hepatitis B, HBsAg level is highest during the immune-tolerant phase and declines through the immune-clearance and immune-control phases (309). In this context, HBsAg level has been evaluated as a biomarker for the natural history of disease, with a specific focus on risk of disease progression.

The level of HBsAg may differentiate the risk of immune escape among patients in the immune-control phase of CHB. In one cohort of 209 genotype-D patients with inactive hepatitis B (310), a combination of HBsAg less than 1000 IU/ml and HBV DNA less than 2000 IU/ml predicted maintenance of an inactive carrier state at 3 years with an 87.9% positive predictive value. This finding has been validated in a Taiwanese study of genotype-B and -C patients (311).

Low levels of HBsAg in the immune-control phase of CHB have also been linked to likelihood of HBsAg seroclearance. The Elucidation of Risk Factors for Disease Control or Advancement in Taiwanese Hepatitis B Carriers (ERADICATE-B) study identified HBsAg levels that strongly predicted HBsAg loss over time (312). Among 688 HBeAg-negative patients, with an HBV DNA less than 2000 IU/ml at baseline, HBsAg clearance occurred at a rate of 1.6% per year. An HBsAg level of less than 10 IU/ml was associated with an annual clearance rate of 7%, 13.2 times that of patients with a baseline HBsAg over 10,000 IU/ml.

HBsAg levels were more useful for predicting long-term HBsAg seroclearance than were HBV DNA levels.

HBsAg levels have been associated with risk of HCC development. Data from 3411 HBV carriers in the REVEAL study showed that both HBsAg and HBV-DNA levels were independent predictors of HCC development. The multivariate-adjusted hazards ratio for HCC development increased from 1.0 (reference) for serum levels of HBV-DNA/HBsAg of <2000/<100 IU/ml to 9.22 (95% CI: 4.34–19.58) for serum levels of HBV-DNA/HBsAg of ≥2000/≥100 IU/ml (313). Among 2688 noncirrhotic CHB individuals, followed for a mean of 14.7 years, the independent predictors of HCC risk were HBV DNA level (HR: 4.7; 95% CI: 2.2–10.0), increased quantitative HBsAg (HR: 7.2; 95% CI: 1.8–28.6), and elevated ALT level (HR: 6.6; 95% CI: 2.2–19.8) (311). HBsAg levels were most useful in patients with an HBV-DNA level <2000 IU/ml; the risk of HCC for individuals with HBV DNA <2000 IU/ml and HBsAg ≥1000 IU/ml was much higher than those with HBV-DNA <2000 IU/ml and HBsAg <1000 IU/ml (HR: 13.7; 95% CI: 4.8–39.3) (311).

HBsAg levels have also been proposed as a biomarker for responsiveness to peginterferon treatment (see the Treatment section). The value of HBsAg levels for decision-making in the context of peginterferon therapy has recently been acknowledged in clinical guidelines (314).

Novel Diagnostic Tests

Quantitative HBeAg Assays

One of the drawbacks to measuring intrahepatic HBV intermediates is the requirement to use liver tissue. However, a correlation exists between the levels of circulating HBsAg and the levels of intrahepatic HBV cccDNA (315). Sensitive quantitative assays for HBsAg, as well as HBeAg, have been developed to study CHB patients (302,316–318), and much more relevant data can be obtained than is suggested by standard qualitative serology. The use of these assays may allow fine-tuning of treatment protocols, particularly with regard to interferon-based therapy (see below).

The therapeutic endpoint for HBeAg-positive CHB is HBeAg seroconversion. A potential role for quantitative HBeAg titer in guiding management algorithms is emerging. A large study using pegylated interferon-α for the treatment of HBeAg-positive CHB found that seroconversion was more likely to occur in the setting of a low HBeAg titer pretreatment, or if there was a rapid decline of HBeAg titer on therapy pretreatment (319). Conversely, the failure of HBeAg levels to fall on treatment predicted nonresponse. In this study the predictive power of HBeAg titer was greater than for HBV viral load, measured using a sensitive PCR assay. Beyond interferon-based therapy, there may be a particular application of HBeAg titer during nucleos(t)ide analogue (NA) therapy, both for predicting seroconversion early, as well as for monitoring patient response. NA therapy rapidly suppresses HBV viral load to undetectable levels, decreasing the utility of HBV DNA as a monitoring tool for HBeAg seroconversion. HBeAg titer has been shown to predict both seroconversion and nonresponse during LMV therapy (316, 317,320–324). In one study HBeAg titer was also observed to predict LMV resistance prior to virological breakthrough (322). The clinical utility of quantitative HBeAg measurements may be greatest when used in conjunction with virological sequencing of the HBV precore/basal core promoter region. Both basal core promoter and precore variants, which occur as quasispecies in HBeAg-positive CHB, are associated with lower HBeAg titer, and stratification according to the dominant virus may be useful. Quantitative HBeAg serology remains a research tool, however, modeled after the method of Perrillo et al. (320), with the level expressed by establishing a standard curve using the Paul Ehrlich HBeAg reference standard.

Assays for Intrahepatic HBV Replicative Intermediates

Assays to quantify the various intrahepatic HBV replicative forms, in particular the HBV cccDNA, total intrahepatic HBV DNA (RC DNA), and pregenomic RNA (pgRNA), could provide insights into the natural history of HBV infection and therapeutic response.

Patients with HBeAg-negative CHB have lower levels of cccDNA than their HBeAg-positive counterparts (303, 304, 325, 326). Further, virion productivity, defined as the ratio of RC DNA to cccDNA, is reduced in HBeAg-negative CHB (Fig 7). This reduced replicative activity is associated with lower levels of pgRNA, implying transcriptional downregulation. In the context of antiviral therapy, preliminary data suggest that cccDNA level pretreatment may predict antiviral response and that significant decline of cccDNA to low end-of-treatment levels may predict durable off-treatment viral suppression, indicating clearance of infected cells (327, 328).

Eradication of HBV cccDNA may not be necessary to control chronic hepatitis B if its replicative activity, as measured by the pgRNA level, is low or quiescent. At present these assays remain in the research domain.

Hepatitis B Core-Related Antigens (HBcrAg)

In an effort to design a simple monitoring tool for patients on antiviral therapy for HBV, a sensitive enzyme immunoassay was developed to detect hepatitis B core-related antigens (HBcrAg) (329), defined as the protein products of precore/core transcription, namely HBeAg and HBcAg. The HBcrAg assay correlates with serum HBV viral load (329, 330). Using this investigative assay, the levels of HBcrAg correlate with viral intrahepatic replicative intermediates (331, 332). If further validated, the HBcrAg assay may provide a simple tool to monitor the level of viral replication in the liver without the need for a liver biopsy.

Ultrasensitive Immunoassays for Anti-HBs and Anti-HBe

The presence of large amounts of HBsAg and HBeAg in the serum may affect the ability to detect circulating antibodies and may obscure the onset of seroconversion. The available commercial assays usually detect anti-HBs and anti-HBe antibodies only after the respective antigens have been cleared from the serum. More sensitive immunoassays can detect antibody in the presence of excess serum antigen (333). Such approaches identified serological responses in the context of active viral replication. For example, all patients with "active" CHB and the majority of patients with immunotolerant CHB demonstrate ongoing humoral immune responses, including anti-HBe and anti-surface antibody (anti-HBs) production. In fact, anti-HBe seroconversion can occur many years (up to 6 years) before the actual loss of HBeAg or onset of liver injury. Similarly, anti-HBs may coexist with virions and subviral HBsAg particles for many years before viral clearance and loss of HBsAg. A small study utilizing a novel assay to detect HBsAg/anti-HBs complexes demonstrates this coexistence and a possible use

in predicting on-treatment response (334). The concept of a relatively nonoverlapping seroconversion from HBeAg-positive to anti-HBe positive status during CHB may need reconsideration (Fig 7).

HBV RNA Assays

It has been known for some time that HBV RNA is found in the circulation of CHB patients (335). How RNA enters the serum compartment, and its biologic function, if any, is unknown. The utility of HBV-RNA measurement as a predictive tool for HBeAg seroconversion while the patient is on nucleos(t)ide therapy was recently examined by designing a specific real-time PCR assay, which used rapid amplification of complementary DNA techniques (336). Both full-length, polyadenylated RNA, as well as a truncated form, were detected. This study showed that reductions in RNA levels at 6 months on the order of 1.8 ± 1.4 $\log_{10}$ copies/ml and 3.1 ± 1.7 $\log_{10}$ copies/ml for the full-length and truncated forms, respectively, were predictive of HBeAg seroconversion after 24 months of NA treatment (336). While clearly in the preliminary stages of investigation and validation, this novel assay shows promise in identifying those who may achieve HBeAg seroconversion.

PREVENTION

Effective strategies for the prevention of HBV infection include 1) the avoidance of high-risk behavior and the prevention of exposure to blood or bodily fluids; 2) active immunization with the hepatitis B vaccine; 3) active-passive immunization with vaccine and hepatitis B immunoglobulin after a suspected high-risk exposure; and 4) nucleos(t)ide therapy for expectant mothers to reduce perinatal transmission rates (discussed previously).

The global HIV epidemic has led to widespread education campaigns directed at decreasing the transmission of bloodborne viruses, including HBV, HCV, and HIV. Changes in sexual practice, the increased use of condoms, and needle-exchange programs all appear to have reduced the incidence of HBV infection. The screening of blood and blood products has led to a dramatic decline in transfusion-acquired HBV infection. The improved disposal of needles and other sharp objects in the hospital setting, as well as the advent of new devices designed to decrease inadvertent needlestick injury, have reduced occupational exposures. Unfortunately, such primary preventative measures are likely to be less effective in countries with a high prevalence of disease, where perinatal or early horizontal infection is common. In these areas, immunoprophylaxis, both passive and active, is the most effective strategy.

Immunization

WHO has recommended that all countries provide universal HBV vaccination programs for infants and adolescents, with appropriate catch-up programs (211). In addition, persons at high risk should be targeted (including persons at occupational risk, institutionalized persons, dialysis patients, recipients of blood products, household members, and sexual partners of CHB patients, travelers to endemic areas, persons who have more than 1 sexual partner in a 6-month period, men who have sex with men, injecting drug users, and prisoners) (337). Prevaccination testing is not cost-effective in low prevalence areas and is only useful in adults from endemic areas to identify those who are either infected or immune and therefore do not require vaccination.

Effective hepatitis B vaccines have been available since 1981. Multiple formulations are now licensed, as either a monovalent vaccine or in fixed combination with other vaccines (including hepatitis A, *Haemophilus influenzae*, diphtheria, tetanus, pertussis, and poliomyelitis). All licensed hepatitis B vaccines are recombinant and are typically made by incorporating the SHBs gene of HBV into the yeast expression vector *Saccharomyces cerevisiae* to generate recombinant HBsAg. They therefore contain the S epitope (the major antigenic determinant or "a" determinant), but not the Pre-S1 or Pre-S2 epitopes. Plasma-derived vaccines are no longer used or recommended. Third-generation recombinant vaccines containing 2 or 3 surface epitopes (S, Pre-S2 ± Pre-S1) have been generated from animal cell lines. They may provide enhanced immunogenicity and therefore a strategy in nonresponders to the standard recombinant yeast-derived vaccine (49, 338, 339). Currently licensed vaccines are, however, very effective at raising protective humoral immunity directed against the HBsAg (337). A new HBV vaccine utilizing a TLR-9 agonist as the adjuvant has been shown to have noninferiority to currently approved vaccine formulations with the requirement for fewer doses (340, 341). A larger phase III study is being conducted to satisfy USA FDA requirements for registration (NCT02117934).

The standard vaccination schedule consists of three doses of vaccine, given intramuscularly, of 10 to 20 ug in adults and 5 to 10 ug in children. The second dose is given 1 month after the first dose and the third, 6 months after the first dose. It is not necessary to restart the series if there have been prolonged intervals between doses. The complete vaccine series induces protective antibody levels in >95% of infants, children and adolescents (337). The efficacy of the vaccine is almost 100% in immunocompetent people who develop antibody levels of >10 IU/ml. The response declines with increasing age beyond 30 years; protection falls to 90% by the age of 40 years, and, by 60 years, protective antibody levels are achieved in only 65 to 75%. Risk factors for nonresponse include obesity and immunodeficiency.

Postvaccination testing is only recommended in the following groups: infants born to HBV-infected mothers, the immunocompromised (including dialysis and HIV patients), healthcare workers and other persons at occupational risk of exposure, and sexual partners of HBV-infected persons. Nonresponse is defined as the failure to mount an anti-HBs response >10 IU/ml 1 to 6 months after the third dose of vaccine. In nonresponders, 25 to 50% of immunocompetent adults will respond to one additional dose of vaccine. For individuals who remain seronegative after this booster dose, a second series (three additional doses) of the double-dose vaccine should be given; 50 to 60% will seroconvert (342). Those remaining seronegative are likely to be true nonresponders.

The duration of protection is at least 15 years (343, 344). Even if anti-HBs titers decline to become undetectable, an anamnestic response appears to persist, providing protective immunity. A small number of high-risk individuals in whom protective antibody titers have been lost develop markers of HBV infection (anti-HBc). However, in most the infections were asymptomatic and detected by regular blood monitoring in the setting of clinical trials. Routine testing postvaccination and routine booster vaccination is therefore not recommended. Additional information is still needed to establish the need for a booster beyond 15 years after immunization in those who are at high risk for exposure (e.g., health care workers). Boosters may be considered to provide reassurance of protective immunity in these special groups (345).

The hepatitis B vaccine is very safe. Anaphylaxis is rare and is the only serious adverse effect that has been documented. There are no data to support a link with demyelinating disorders, Guillain-Barré syndrome, chronic fatigue syndrome, sudden infant death syndrome, lupus erythematosus, or other disorders (337).

Impact of Vaccination on Transmission

Active-passive immunization was shown in the mid 1980s to prevent >95% of perinatal transmission (346, 347). Taiwan was the first country to introduce universal neonatal vaccination in 1986. Since the program began, the prevalence of HBsAg in children under 5 years of age has decreased from 9.3% in 1984 to 0.5% in 2004 (344). This suggests not only protection of those vaccinated but also prevention of horizontal transmission. Subsequently the incidence of both fulminant hepatitis (348) and HCC in children has declined sharply (349).

Postexposure Prophylaxis

Passive immunization with hepatitis B immunoglobulin (HBIG) provides temporary immunity against HBV infection in those who are not immune. The most common indication for use is for postexposure prophylaxis. Postexposure prophylaxis is the standard of care for all nonvaccinated individuals exposed to infectious blood or bodily fluids, including infants born to HBsAg-positive mothers, and following percutaneous (e.g., needlestick), mucosal, or sexual exposure to HBV. The first dose of HBV vaccination is given within 12 hours of exposure, followed by second and third doses at 1 and 6 months respectively. HBIG (0.06 ml/kg intramuscular) should be administered with the first dose of vaccine and can be repeated at 1 month if there has been no response to the vaccine. Vaccinated individuals with documented response do not require prophylaxis.

MANAGEMENT OF HBV INFECTION

Acute Hepatitis B

Acute hepatitis B (AHB) is a spontaneously resolving infection in the majority of cases in immunocompetent adults. Antiviral therapy is not generally indicated unless fulminant disease is present. Care, in the absence of fulminant disease, is supportive, with the provision of adequate nutrition and hydration and the avoidance of hepatotoxic drugs, such as acetaminophen. However, there has been some interest in whether NA therapy would benefit patients with severe AHB. LMV use in patients presenting to a transplant center with severe AHB, defined by an INR >2.0, significantly improved mortality compared to a historical control; 14 of 17 patients (82.4%) survived with full recovery without liver transplantation compared to a historical cohort in which the survival without liver transplantation was 20% (4/20) (350). In contrast, a randomized controlled trial in India failed to demonstrate a benefit of LMV therapy in severe AHB defined by two of three criteria (hepatic encephalopathy; serum bilirubin ≥10.0 mg/dL [171 μmol/l] and/or [4] INR ≥1.6) (351), although no patient in this study required liver transplantation. Given the limitations of current data, a trial of NA therapy may be reasonable in fulminant HBV with coagulopathy and elevated INR; the risk of resistance associated with long-term LMV would generally favor ETV or TDF. There have been case reports of lactic acidosis associated with ETV used for fulminant hepatitis B infection, but these reports do not prove causality (352).

Chronic Hepatitis B

Principles of Management

The aim of treatment for CHB is the prevention of clinical complications including decompensated liver disease and hepatocellular carcinoma. The prevention of these clinical end points may be achieved by durable suppression of HBV replication (353–358). As clinical end points may take years to eventuate, sustained virological response (SVR) has been adopted as the goal of therapy both in clinical trials and day-to-day practice (230, 359). SVR is defined as a decrease in serum HBV DNA to undetectable levels by PCR assays, with loss of HBeAg in patients who were initially HBeAg-positive (230). HBeAg seroconversion, in the context of HBeAg positive disease, has been shown to correlate with sustained viral suppression and clinical benefit, particularly in patients treated with pegIFN, and is therefore an end point for therapy (360). The end point for therapy in the setting of HBeAg-negative CHB is HBsAg loss (230, 314).

Indications for Therapy

Candidates for antiviral therapy can be classified into three groups: HBeAg-positive CHB, HBeAg-negative CHB, and patients with advanced fibrosis/cirrhosis (who may be either HBeAg positive or negative). The clinical utility of this approach is based on the expected differences in the duration of therapy that will be required for these groups.

HBeAg-Positive CHB

The traditional clinical end point of therapy in HBeAg-positive CHB is HBeAg seroconversion. It is therefore reasonable to offer a trial of therapy to all patients with active disease, defined by a viral load >20,000 IU/ml and either raised ALT >2x the upper limit of normal (ULN) or moderate to severe inflammation on liver biopsy. In patients with an ALT <2x ULN, in the presence of viral replication, the decision whether to commence therapy is influenced by the severity of both inflammation and fibrosis on liver biopsy. Most professional bodies recommend a trial of observation for 3 to 6 months to allow for spontaneous seroconversion before initiating therapy (230, 314, 361). The options for therapy include interferon-based therapy or any of the recommended NA (Table 4A). HBeAg seroconversion is durable following peginterferon therapy, but it may be less durable following withdrawal of NA therapy (362). A period of consolidation therapy with NA for at least 12 months following seroconversion is recommended, and patients must be followed for reactivation.

HBeAg-Negative CHB

The only agreed end point of therapy for patients with HBeAg-negative CHB is HBsAg loss. Sustained virologic response may be achieved in a small percentage of patients using interferon-based therapy; however, it is likely that most patients will require prolonged therapy with a NA. Treatment decisions must therefore weigh the risk of future complications against the long-term financial costs and risk of antiviral resistance. Liver biopsy is useful in decision-making, as the presence of significant fibrosis should trigger consideration of therapy. Treatment is reasonable in patients with a viral load >2,000 IU/ml and evidence of moderate/severe inflammation or significant fibrosis (230). In patients with mild fibrosis and inflammation but ongoing replication, there are no data to determine whether the risk of long-term antiviral resistance outweighs the benefit of initial viral suppression (Table 4B).

TABLE 4A Responses to approved antiviral therapies among treatment-naive patients with HBeAg-positive

	Placebo/ Control Groups from Multiple Studies	Standard IFN-α 5 MU qd or 10 MU tiw 12-24 wk	Lamivudine 100 mg qd 48-52 wk	Adefovir 10 mg qd 48 wk	Entecavir 0.5 mg qd 48 wk	Tenofovir 300 mg qd 48 wk	Telbivudine 600 mg qd 52 wk	PegIFNα 180 mcg qw 48 wk	Peg IFNα 180 mcg qw + Lamivudine 100 mg 48 wk
Loss of serum HBV DNA*	0%–17%	37%	40%–44%	21%	67%	76%	60%	25%	69%
Loss of HBeAg	6%–12%	33%	17%–32%	24%	22%	na	26%	30%/34%†	27%/28%†
HBeAg seroconversion	4%–6%	Difference of 18%	16%–21%	12%	21%	21%	22%	27%/32%†	24%/27%†
Loss of HBsAg	0%–1%	7.80%	1%	0	2%	3.2%	0%	3%	3%
Normalization of ALT	7%–24%	Difference of 23%	41%–75%	48%	68%	68%	77%	39%	46%
Histologic improvement	na	na	49%–56%	53%	72%	74%	65%	38%‡	41%‡
Durability of response		80%–90%	50%–80%§	~90%§	69%§	na	~80%	na	na

*Hybridization or branched chain DNA assays (lower limit of detection 20,000-200,000 IU/mL or 5-6 log copies/mL) in standard IFN-α studies and some lamivudine studies, and PCR assays (lower limit of detection approximately 50 IU/mL or 250 copies/mL) in other studies. na = not available.

†Responses at week 48 / week 72 (24 weeks after stopping treatment).

‡Post-treatment biopsies obtained at week 72.

§Lamivudine and entecavir – no or short duration of consolidation treatment, Adefovir and telbivudine – most patients had consolidation treatment.

TABLE 4B Responses to approved antiviral therapies among treatment-naive patients with HBeAg-negative CHB

	Control/Placebo Groups from Multiple Studies	Standard IFN-α 5 Mu qd or 10 MU tiw 6-12 mo	Lamivudine 100 mg qd 48-52 wk	Adefovir 10 mg qd 48 wk	Entecavir 0.5 mg qd 48 wk	Telbivudine 600 mg qd 52 wk	Tenofovir 300 mg qd 48 wk	Peg IFNα 180 mcg qw 48 wk	PegIFN-α 180 mcg qw+ Lamivudine 100 mg qd 48 wk
Loss of serum HBV DNA*	0%–20%	60%–70%	60%–73%	51%	90%	88%	93%	63%	87%
Normalization of ALT	10%–29%	60%–70%	60%–79%	72%	78%	74%	76%	38%	49%
Histologic improvement	33%	na	60%–66%	64%	70%	67%	72%	48%	38%†
Durability of response	Control	10%–20%	<10%	~5%	3%	na	na	~20%	~20%

na = not available

*Hybridization or branched chain DNA assays (lower limit of detection 20,000-200,000 IU/mL or 5-6 log copies/mL) in standard IFN-α studies and some lamivudine studies, and PCR assays (lower limit of detection approximately 50 IU/mL or 250 copies/mL) in other studies.

†Post-treatment biopsies obtained at week 72.

Adapted from Lok and McMahon (230).

Cirrhosis

Sustained viral suppression improves clinical outcomes in the setting of CHB and advanced liver disease, in particular, reducing the risk of HCC (353, 363). Long-term NA therapy would normally be recommended. It is critical that these patients be monitored closely both for initial virological response and, subsequently, for the development of antiviral resistance, to allow institution of salvage therapy before the serum ALT becomes elevated. Patients with compensated cirrhosis and HBeAg-positive CHB can be considered for pegylated-interferon with close monitoring for a hepatitis flare and long-term monitoring for virological relapse (364). Patients with decompensated liver disease should not receive interferon-based therapy because a hepatitis flare in this setting is associated with a high chance of mortality; rather, long-term NA therapy and referral to a transplant center is indicated.

Treatment of Special Populations

HIV-HBV Coinfection

Since antiretroviral therapy is recommended for all HIV-infected patients, HBV-coinfected patients need to be identified and treated with a regimen effective for both viruses, with TDF and emtricitabine (FTC) being the favored combination (365). In the rare coinfected patients declining antiretroviral therapy, HBV therapy should be based on agents that will not select for HIV drug resistance.

ETV is now recognized to have anti-HIV activity and is associated with the accumulation of HIV-type 1 variants with the LMV resistance mutation M184V (366). Therapeutic options therefore include adefovir dipivoxil (ADV) (10 mg) daily or pegIFN. Although Telbivudine (LdT) does not target HIV, its use as monotherapy is not recommended because of the risk of selection of the M204I mutation in the YMDD motif.

The hepatitis B vaccine should be given to all HIV-positive persons who are negative for HBV seromarkers. The vaccine should be given when CD4 cell counts are ≥200 cells/μl, as response is poor below this level. Persons with CD4 counts below 200 cells/μl should defer HBV vaccination until antiretroviral therapy has reconstituted CD4 counts above this threshold.

Pregnancy

The introduction of active-passive immunization of infants born to HBV-infected mothers has significantly reduced the rate of perinatal transmission where this therapy is available (367). However, even with timely administration, highly viremic mothers (≥ 6 $\log_{10}$ copies/ml) retain a >10% transmission rate to their newborns (368). Therefore, this group of patients will benefit from NA therapy to reduce viral load, in order to augment the efficacy of immunization. A meta-analysis of ten randomized, controlled trials of active-passive immunization, with or without LMV, examined outcomes for over 450 patients. Nine studies commenced therapy at 28 weeks of gestation, whereas one study commenced at 32 weeks. The pooled odds ratio (OR) for infant infection, as determined by HBsAg positivity at 9 to 12 months of age, was 0.31 (CI: 0.15–0.63) favoring LMV (369). A second meta-analysis of six studies examining the use of LdT in similar settings again strongly favored its use with a pooled OR of 0.11 (CI: 0.04–0.31) (370). A recent prospective, open-label, case-control study from China (371) on 362 LdT-treated pregnant women (either second or third trimester) found no cases of vertical transmission, as opposed to a 9.3% transmission rate in the 92 controls ($P < 0.001$) (371). No obstetric complications or congenital malformations were reported.

TDF is the preferred agent in pregnancy and has been recommended in major society clinical practice guidelines to treat this population, given its high potency and ability to rapidly reduce viral load with a shorter duration of therapy (314, 361). To investigate this, a recent Australian "opt-in" prospective study examined the use of TDF as compared with LMV and placebo in pregnant patients with high viral load (372). The patients were predominantly of Asian ethnicity (88.5%), and those who elected to be treated commenced therapy in the third trimester. All infants received active-passive immunization at birth. Significantly more patients achieved a viral load <6 $\log_{10}$ copies/ml in the TDF group when compared to LMV (18% vs. 3%, $P = 0.01$). Despite this difference, the rate of perinatal transmission was similar between the two groups (2% TDF vs. 0% LMV) but significantly better than the untreated group (20% transmission rate) (372). The USA FDA classifies both LdT and TDF as pregnancy category B drugs (no congenital risk in animal studies, but unknown data in humans), whereas LMV, ADV, and ETV are category C (teratogenic in animals, but unknown in humans). Therefore, either LdT or TDF are drugs of choice. However, more long-term data on the safety of TDF are currently available from its use in the HIV population. The Antiretroviral Pregnancy Registry has reported data on 2,330 live births having had TDF exposure during the pregnancy. A congenital malformation rate of 2.3% was found in infants delivered to these mothers, which is equivalent to the baseline general population rate (373).

An issue that arises in patients who started antiviral therapy before parturition is when or if to stop treatment. Typically, given prescribing restrictions, these patients would not otherwise have been eligible for treatment and therefore are unlikely to qualify for ongoing prescription. A concern with ceasing therapy soon after delivery is the risk of a postpartum flare of hepatitis, due to a combination of increased viral replication and immune reconstitution in the puerperium. One small study has shown, however, that extending the duration of NA therapy beyond delivery does not abrogate the risk of flare (374). Further studies will be required to shed light on this area of management.

Immunosuppression

Immunosuppression is a unique situation in which antiviral therapy is indicated for HBV. Reactivation of HBV replication with increase in ALT levels has been reported in 20 to 50% of HBsAg-positive patients undergoing immunosuppression or cancer chemotherapy (375–377). Such patients often have a complex quasispecies mix that harbors mutations in the major hydrophilic region, thus making immune escape more likely (378). The risk is especially high in patients undergoing hematopoietic stem cell or solid organ transplantation or with treatment with the anti-CD20 monoclonal antibody therapies rituximab and ofatumumab, particularly in nonHodgkin lymphoma patients (212,379–381). There are also reports of reactivation following transarterial chemo-embolization for HCC and anti-TNF therapies for inflammatory bowel disease and rheumatoid arthritis (377, 382, 383). Although most cases are asymptomatic, icteric flares, hepatic decompensation, and death have all been reported. Patients with a high pretreatment viral load, defined by HBeAg-positivity or HBV DNA >60,000 IU/ml, are at higher risk of reactivation. The degree of risk can be estimated by combining the degree of immunosuppression expected from the treatment regimen with the patient's serology (Table 5) (213). Therefore, all patients who are to undergo an immunosuppressive treatment regimen should have serology for HBV assessed, including a

TABLE 5 Estimated risk of HBV reactivation based on serologic profile and type of therapeutic immunosuppression

	HBsAg-Positive	HBsAg-Negative/Anti-HBc Positive
Anti-CD20	30–60%	>10%
Anthracyclines	15–30%	1–10%
Corticosteroids (≥4 weeks & ≥10 mg)	>10%	1–10%
TNFα inhibitors	1–10%	1%
Other cytokine/integrin inhibitors[a]	1–10%	1%
Tyrosine kinase inhibitors	1–10%	1%
Corticosteroids (≥4 weeks & <10 mg)	1–10%	<1%
Corticosteroids (≤1 week)	<1%	<< 1%
Traditional immunosuppression[b]	<1%	<< 1%

(Adapted with permission from Perrillo et al. [213]).
[a]Abatacept, ustekinumab, natalizumab, vedolizumab.
[b]Azathioprine, 6-mercaptopurine, methotrexate. High risk group >10%; moderate risk group 1–10%; low risk group <1%.

HBV DNA if HBsAg-negative/anti-HBc positive. All nonimmune individuals should be vaccinated.

Prophylactic therapy with LMV reduces the rate of HBV reactivation, severity of associated hepatitis, and mortality (377,384–387). HBsAg-positive patients with baseline viral loads less than 2,000 IU/ml should be started on LMV at the onset of immunosuppression. Patients with baseline HBV DNA levels >2,000 IU/ml should continue antiviral therapy until a therapeutic end point is reached, as viral relapse has been reported after withdrawal (388). In this setting, where therapy may be prolonged, a high potency NA may be more appropriate.

Patients who are HBsAg-negative and anti-HBc ± anti-HBs-positive (i.e., occult hepatitis B) remain at risk of reactivation of HBV when profoundly immunosuppressed. This is due to persistence of the cccDNA template. Reactivation is infrequent but seen primarily with anti-CD20 therapies. Use of these agents mandates prophylactic treatment (214). In other situations, it may be reasonable to monitor patients closely (every 1 to 3 months) and to initiate treatment when serum HBV DNA level becomes detectable, ideally before a hepatitis flare is diagnosed.

Therapy should be continued for at least 12 months after completion of chemotherapy (230, 314, 361). Even after cessation of successful NA prophylaxis, patients treated with anti-CD20 therapy may require longer term regular monitoring in case of delayed reactivation (389).

ANTIVIRAL THERAPY

As highlighted previously, there are two classes of therapy currently available for the management of CHB:

1) Nucleos(t)ide analogues (NA)
2) Pegylated interferon-α

Nucleos(t)ide Analogues

The development of potent oral NA that are safe, well tolerated, and have a low risk of antiviral resistance represent a major therapeutic advance (Tables 5A and 5B). There are five NA currently approved for the management of CHB—LMV, LdT, ADV, ETV, and TDF—which belong to three separate drug classes. It is useful to consider these drugs within their class due to overlapping resistance profiles. The use of a sixth agent, FTC, is approved, in combination with TDF, only for HIV infection, but FTC has some efficacy in treating HBV. ETV and TDF are now considered first line agents for CHB due to their high potency and high genetic barrier to resistance.

The advantages of NA are ease of administration, rapid viral suppression, and a good safety profile. If SVR is maintained, then long-term therapy results in improved clinical outcomes. The major disadvantage is the requirement for prolonged therapy in the majority of patients, the potential for drug resistance, and the absence of robust safety data for these agents during conception and pregnancy. The latter point is especially important for younger patients in whom prolonged therapy may be required. NA are also the only therapeutic option for patients with decompensated liver disease before liver transplantation.

The HBV cccDNA pool provides a difficult obstacle for antiviral therapy to overcome. As HBV replication does not employ a semiconservative mechanism, any nucleos(t)ide analogue-based therapy can only indirectly affect the preexisting cccDNA template. The likely reason for relapse, seen after completion of antiviral therapy for hepatitis B infection, is the persistence of the HBV cccDNA, which can re-establish pretreatment replication levels.

L-Nucleoside Analogues

Lamivudine (LMV)

Lamivudine is the (-) enantiomer of 2′, 3′ dideoxy-3′-thiacytidine. It inhibits DNA synthesis by the incorporation of its active triphosphate site into the growing DNA chain, resulting in chain termination, and was the first agent to be approved for the treatment of CHB. Unfortunately, the use of LMV is frequently complicated by the emergence of drug resistance (see below). As a result, LMV monotherapy is no longer recommended for treatment naïve patients. For economic reasons, however, it is likely that widespread use will continue in the developing world.

LMV is not a potent inhibitor of HBV replication. Treatment results in a mean decline of serum HBV DNA of 4- to 5-$\log_{10}$ copies/ml by 48 weeks. In HBeAg-positive patients, HBV DNA levels became undetectable in 40 to 44% by 48 to 52 weeks, 41 to 75% normalized ALT, and histological improvement was noted in 49 to 56% (compared to 23 to 25% of placebo controls). HBeAg seroconversion has occurred in 16 to 18% (compared to 4% to 6% of controls, respectively) (390–394). HBeAg seroconversion increases with prolonged duration of therapy up to 50% by 5 years (393–396). Not surprisingly, pretreatment ALT is the strongest predictor of response in HBeAg-positive patients—seroconversion was greatest in patients with baseline ALT >5 x the upper limit of normal (ULN) in clinical trials and progressively less frequent in patients with ALT levels 2 to 5 x ULN, 1 to 2 x ULN and normal ALT (47%, 21%, 9%, and 2%, respectively) (397, 398).

Following treatment withdrawal, HBeAg seroconversion was demonstrated to be durable in 50 to 80% of patients in clinical trials, but real-world studies suggest that durability is much lower, with relapse eventually occurring in the majority of patients (362, 399, 400). If LMV is used, it is recommended that therapy be continued for at least 12 months after seroconversion to minimize the risk of viral reactivation following treatment withdrawal (401). LMV is also partially effective in HBeAg-negative patients (402–406); 1 year of therapy will suppress HBV DNA to undetectable levels in 60 to 70% of patients using sensitive PCR assays (404, 405, 407, 408). This is unlikely to reflect increased potency, as compared to HBeAg-positive cohorts, but rather a lower baseline viral load in the HBeAg-negative state. Despite apparent effective viral suppression, the vast majority of patients relapsed (90%) when treatment was stopped (403). Long-term therapy is therefore required for sustained viral suppression. Unfortunately, prolonged therapy is complicated by the progressive development of LMV resistance. In one large study of HBeAg-negative patients, virologic remission decreased from 73% at 12 months to 34% at 48 months, due to the emergence of LMV resistant mutants (Table 6) (354). These rates are even less encouraging in HIV coinfected patients.

One seminal, double-blind, randomized, placebo-controlled trial of LMV therapy (353) in 651 Asian patients, who had evidence of active viral replication and bridging fibrosis and cirrhosis on liver biopsy, found a significant reduction in overall disease progression (as defined by an increase in the Child-Turcotte-Pugh score or hepatic decompensation) and HCC development (353). The clinical benefit was observed mainly in the 51% of patients who did not experience virologic breakthrough, emphasizing

TABLE 6 Cumulative annual incidence of antiviral resistance in differing patient populations

Treated Population	Percentage of Patients Resistant after Years of Therapy					
	1	2	3	4	5	6
LAM	23	46	51	71	80	-
LdT HBeAg-Pos	4.4	21	-	-	-	-
LdT HBeAg-Neg	2.7	8.6	-	-	-	-
ADV HBeAg-Neg	0	3	6	18	29	-
ADV (LAM-resistant)	≤20%	-	-	-	-	-
TDF	0	0	0	0	0	0
ETV (naïve)	0.2	0.5	1.2	1.2	1.2	1.2
ETV (LAM-resistant)	6	15	36	46	51	57

(Adapted with permission from Zoulim and Locarnini [468]).

both the importance of sustained viral suppression and the problems associated with LMV resistance. Prospective data on clinical outcomes in patients with less advanced disease are lacking, although retrospective analyses suggest improvement in patients maintaining viral suppression after starting therapy with LMV (354, 355). Early on, LMV therapy demonstrated benefit in the management of patients with decompensated cirrhosis, but it has since been supplanted by newer agents (409–412). Clinical improvement takes 3 to 6 months, and the risk of HCC persists, so that treatment should be initiated early and surveillance for HCC continued. In the original LMV registrational study in patients with advanced fibrosis or cirrhosis, durable viral suppression decreased the cumulative incidence of HCC from 7.4% to 3.4% ($P = 0.047$) by 32 months of therapy (353), despite the confounding effect of antiviral resistance emergence. More recently, ETV and TDF have been confirmed to reduce the risk of HCC, particularly in cirrhotic patients (413–416).

Overall, LMV is very well tolerated. Various mild adverse events have been reported in patients receiving LMV, but all were observed to occur at the same frequency among controls (230).

Telbivudine (LdT)

Telbivudine is an L-nucleoside with a potent anti-HBV effect; 48 weeks of therapy will reduce HBV viral load by 6 $\log_{10}$ copies/ml. In clinical trials, LdT has been shown to be more potent than LMV in suppressing HBV replication in both HBeAg-positive and negative CHB (417), which was sustained in those patients who experienced an early response (418). A HBV DNA reduction to less than 5 $\log_{10}$ copies/ml, defined as the primary endpoint, was achieved by 75.3% versus 67.0% of patients receiving LdT as compared to those receiving LMV (417) and was associated with histologic improvement as well. Longer term follow-up studies of this phase III study cohort have shown an HBeAg seroconversion rate of 37% after 3 years of therapy (419). This study also showed that 84% of patients who stopped therapy had an undetectable viral load after 52 weeks. Data regarding durability of response are not available beyond this time point. Despite the potent antiviral effect, LdT is also associated with a high rate of resistance (25.1% and 10.8% at 2 years in HBeAg-positive and -negative, respectively), conferred by mutations in the YMDD motif (420). Therefore, LdT resistance mutations are cross-resistant with LMV. For this reason LdT monotherapy has a limited role in the treatment of HBV infection.

Acyclic Nucleoside Phosphonates (Nucleotide Analogues)

Adefovir Dipivoxil (ADV)

Adefovir dipivoxil is the orally bioavailable prodrug of ADV, a nucleotide analogue of adenosine monophosphate. Similar to other NAs, it inhibits HBV DNA polymerase by acting as a chain terminator. It can inhibit both reverse transcriptase and DNA polymerase activity. The degree of HBV suppression achieved is less than that with LMV therapy, with 48 weeks of therapy achieving a mean viral load decline of 3 to 3.5 $\log_{10}$ copies/ml (421). Furthermore, approximately 30% of NA-naïve patients are primary nonresponders (defined as a <2 $\log_{10}$ drop in VL by 6 months of treatment) (422). ADV has activity against both wild type and LMV-resistant HBV *in vitro* and clinically.

In the phase III registrational trial of ADV monotherapy for HBeAg-positive CHB, 10 mg daily of ADV for 48 weeks was associated with significant benefits in mean HBV DNA reduction (3.5 $\log_{10}$ copies/ml vs. 0.6, $P < 0.001$), ALT normalization (48% vs. 16%, $P < 0.001$) and histologic response (53% vs. 25%, $P < 0.001$) compared to placebo (423). HBeAg seroconversion occurred in 12% at 48 weeks versus 6% in placebo ($P < 0.049$), and was more common in patients having a higher baseline ALT. Cumulative seroconversion rates increased with continued therapy to 48% after 5 years (424). No difference in response was observed by racial background or viral genotype. At a dose of 30 mg per day for 48 weeks, ADV was associated with a superior clinical effect compared to the 10-mg dose but also with an 8% incidence of nephrotoxicity, including the rare development of Fanconi syndrome. The ADV dose approved for clinical use was consequently limited to 10 mg per day. Suboptimal dosage and pharmacogenomic factors may therefore be contributing to the primary nonresponse rate (425). HBeAg seroconversion appears to be durable, being maintained in 92% of 76 patients, who were followed for a median of 52 (5 to 152) weeks off treatment after a median 80 (30 to 139) weeks of therapy (230). Patients were treated for a median of 41 weeks postseroconversion, which may explain the high durability.

ADV is also effective in the management of HBeAg-negative CHB. In comparison to placebo, 48 weeks of ADV was associated with a higher rate of undetectable HBV DNA by sensitive PCR assay (51% vs. 0%, $P < 0.001$), ALT normalization (72% vs. 29%, $P<0.001$), and histologic response (64% vs. 33%, $P<0.001$) (426). The rate of viral suppression increased with time to 67% after 240 weeks (427). Although the relapse rate is very high (92%) in patients with HBeAg-negative CHB if therapy is stopped after 48 weeks (428), it may be possible to withdraw therapy after long-term viral suppression. In a cohort of 33 patients, who maintained virological suppression on ADV for a median of 4 to 5 years, 67% maintained biochemical remission to a median follow-up of 18 months (range 15 to 20). Although all experienced SVR, HBV DNA levels remained at relatively low levels similar to the inactive HBsAg carrier state ($<50{,}000$ copies/ml) and, in most cases, declined over time. Persistent biochemical relapse was successfully treated by reinstitution of ADV in 3% of patients (427).

The risk of ADV nephrotoxicity and the observed primary nonresponse rate make ADV less suitable as a therapy for patients with advanced liver disease, either compensated or decompensated. While ADV has been shown to be effective as salvage therapy in decompensated CHB complicated by LMV resistance (429), TDF is the preferred agent in this setting.

Although less potent as an antiviral agent compared to LMV, ADV offers the benefit of a reduced incidence of drug resistance. Furthermore, as the main resistance profile of ADV differs from LMV, these two drugs do not suffer from cross-resistance (Table 7).

Tenofovir Disoproxil Fumarate (TDF)

Tenofovir is one of the two first-line NAs recommended for the treatment of CHB. It is structurally similar to ADV, and these two drugs are equipotent *in vitro*. However, because TDF is associated with less nephrotoxicity, pharmacokinetic studies determined a safe dose 30 times greater (300 mg per day) than ADV. This explains the much greater potency seen in clinical trials (6 to 7 $\log_{10}$ copies/ml reduction in viral load) (421). TDF has activity against both HIV and HBV. It is currently approved as both monotherapy and combination therapy with FTC for the management of HIV and HIV/HBV coinfection. TDF/FTC combination therapy is currently recommended as first-line therapy for HBV-HIV coinfection (430). It is not approved for the management of HBV mono-infection, although it does hold the promise of potent viral suppression and a high resistance barrier.

The superior efficacy of TDF, compared to ADV, in the management of CHB was established in two phase III studies, one examining HBeAg-positive and the other HBeAg-negative infection (431). Both studies treated patients for 48 weeks (200 mg daily TDF versus 10 mg daily ADV) with a primary endpoint of complete virologic response (HBV DNA <400 copies/ml) plus histologic response (two point reduction in Knodell inflammatory score). In the study of HBeAg-positive patients, which enrolled a predominantly Caucasian population (51.5% Caucasian; 36% Asian), with a relatively even distribution of the four major genotypes, 66% of TDF-treated patients reached the primary endpoint compared to 12% of placebo ($P < 0.001$) (431). TDF was also superior with regards to ALT normalization (68% vs. 54%, $P = 0.03$), HBsAg loss (3.2% vs. 0%, $P = 0.02$), and HBV DNA suppression to <400 copies/ml on intention-to-treat analysis (76% vs. 13%, $P < 0.001$). No statistically significant differences were seen in histologic improvement alone or HBeAg seroconversion (21% vs. 18%, $P = 0.36$) (431).

The HBeAg-negative trial also had a predominantly Caucasian population (64.5% Caucasian; 24.5% Asian), with a preponderance of genotype D infection (63.5%). The primary endpoint was reached in 71% of TDF-treated compared to 49% of placebo-treated patients ($P < 0.001$) (431). Similar to the HBeAg-positive group, this significance was driven by complete viral suppression, attained in 93% vs. 63% ($P < 0.001$) of patients without a significant difference in histologic response. There was no difference in ALT normalization (431).

Of the 641 initially randomized patients, 437 (68.2%) completed a further 7 years as part of a long-term treatment study (432). Complete viral suppression (HBV DNA <400 copies/ml) was maintained in 99.3% of the cohort. At enrollment into the long-term study, 154 patients were HBeAg-positive. At the end of follow-up, 54.5% had lost HBeAg, and 11.8% had lost HBsAg. Only one patient in the HBeAg-negative group lost HBsAg (0.3%) (432). No resistance to TDF was detected (433). Baseline and follow-up biopsies were available for 348 patients after 5 years of treatment. Regression of fibrosis (reduction of at least one point in Ishak fibrosis score) was seen in 51% of these patients (271). Additionally, while 96 of these patients were deemed cirrhotic on baseline biopsy, remarkably, 74% of these had regressed to noncirrhotic scores at follow-up (271).

Although generally well tolerated, TDF therapy may be associated with renal insufficiency, and there have been rare case reports of reversible Fanconi syndrome (434). No cases of Fanconi syndrome have been seen in long-term follow-up studies (432, 435), and elevations of serum creatinine ≥0.5 mg/dL were seen in only 1.7% of patients after 7 years (432). The risk of TDF-induced Fanconi syndrome may be related to rare variants in multiple genes involved in renal drug handling or renal cell homeostasis, but no biomarker has yet been determined (436). TDF has been associated with bone loss and osteomalacia in the HIV-treated population, for whom TDF has been available since 2001 (437). This has been postulated to result from hypophosphatemia induced by renal proximal tubular dysfunction, similar to the mechanism for Fanconi syndrome (438). In a study examining this issue, TDF was independently associated with a reduction in bone density in addition to traditional risk factors for osteoporosis (439). However, this was a modest reduction (OR 2.95 CI [1.14–7.45], $P = 0.026$), and limited to the hip. The authors of this study concluded that classic risk factors were more important in risk-stratifying patients taking TDF (439). TDF is effective for the management of both LMV- and ADV-resistance (see below).

Tenofovir Alafenamide (TAF)

Tenofovir alafenamide is the orally bioavailable phosphonamidate prodrug of tenofovir. In comparison with TDF, TAF enables enhanced delivery of the parent nucleotide and its active diphosphate metabolite into lymphoid cells and

TABLE 7 Patterns and pathways of antiviral drug resistance in chronic hepatitis B in the context of cross-resistance

Pathway	Primary Resistance Mutation/s	LAM	LdT	ETV	ADV	TDF
Wild-type	-	S	S	S	S	S
l-Nucleoside	M204I/V ±204I/V	R	R	I	S	S
Acyclic phosphonate	N236T	S	S	S	R	I
Shared	A181T/V	R	R	S	R	I
Double	A181T/V + N236T	R	R	S	R	R
d-Cyclopentane	L180M + M204V/I ± I169T ± T184G ± S202I/G ± M250V	R	R	R	S	S

(Adapted with permission from Liaw et al. [361]).

hepatocytes. Hydrolysis of TAF occurs by action of the enzyme carboxylesterase-1, which is predominantly expressed in HBV-infected hepatocytes (440). The TAF formulation lowers plasma exposure by 90% and has been designed to minimize the bone and renal toxicity associated with TDF (441). TAF is currently in phase III clinical trial development and is expected to be approved in Europe and North America in 2016 and Asia in 2017.

D-Cyclopentanes

Entecavir (ETV)

Entecavir is the second NA recommended as a first-line agent for the treatment of CHB. ETV is an analogue of 2′-deoxyguanosine. It inhibits HBV replication at three different steps: 1) priming of HBV DNA polymerase; 2) reverse transcription of minus-strand DNA; and 3) synthesis of plus-strand HBV DNA. ETV is more potent than LMV or ADV, reducing HBV DNA by 6- to 7-$\log_{10}$ copies/ml after 48 weeks of treatment (421).

ETV was compared to LMV in the phase III registrational trial for the management of HBeAg-positive CHB (442). At week 48, 0.5 mg daily of ETV versus 100 mg daily of LMV was associated with superior rates of HBV DNA undetectability (67% versus 36% by sensitive PCR assay, $P < 0.001$), normalization of ALT (68% vs. 60%, $P = 0.02$), and histologic improvement (72% vs. 62%, $P = 0.009$). HBeAg seroconversion was similar (21% vs. 18%, $P = 0.33$) and was more common in patients having a higher baseline ALT. Rates of undetectable DNA and normal ALT continued to increase through to 96 weeks (443). In those patients who stopped therapy at this point, 5.1% vs. 2.8% had lost HBsAg 24 weeks after cessation in those taking ETV vs. LMV, respectively (444). Studies of long-term maintenance therapy have shown continued efficacy out to 5 years, with an additional 23% of patients achieving HBeAg seroconversion (445). No difference in response was observed by racial background or viral genotype.

In HBeAg-negative CHB, ETV was again associated with improved rates of undetectable HBV DNA, normalization of ALT, and histologic improvement compared to LMV (446). However, of 257 patients who had achieved undetectable HBV DNA and normal ALT levels after 48 weeks of treatment, 97% had detectable DNA within 24 weeks off-treatment, and 51% had elevated ALT levels (447). This reinforces the concept that long-term NA therapy is required for HBeAg-negative infection.

ETV-related viral suppression has a significant impact on regression of histologic fibrosis (272). A subset of 57 patients enrolled in the above studies (41 HBeAg-positive, 16 HBeAg-negative) had paired liver biopsies available for comparison. These were taken after 48 weeks of treatment and after at least 3 years of maintenance therapy (range 3 to 7 years). All patients had HBV DNA <300 copies/ml at the time of the second biopsy and 67% were Asian, hence genotypes B and C were most prevalent (60%). Ninety-six percent of this cohort had a significant reduction in necroinflammation, and 88% had improvements in their Ishak fibrosis score. All 10 patients with cirrhosis had regression and were considered no longer cirrhotic (272).

Concern regarding the safety of ETV in decompensated HBV-related cirrhosis was raised after a series of five patients developed severe lactic acidosis (448). All patients had end-stage liver disease (MELD) scores of >20, indicating severely impaired liver function. One patient died, while treatment withdrawal resulted in resolution of the acidosis in the remaining four. A prospective, randomized study examining the use of ETV, 1 mg, (twice the standard dose), or ADV 10 mg for up to 96 weeks in the decompensated cirrhotic population (449). Forty-nine percent of the ETV group had achieved a HBV DNA <300 copies/ml, compared to 16% in the ADV group at 24 weeks. This increased to 57% vs. 20% by week 48 ($P < 0.0001$ for both time points) (449). Week 24 mortality was 12% in both groups. No cases of lactic acidosis were seen, although no patient had a MELD above 20, and overall safety profile was similar for the two drugs.

In general, ETV is very well tolerated, its safety profile being similar to that of LMV in the registrational trials (442, 446). Preclinical studies in rats, treated with doses 3 to 40 times the maximal human dose, found an increased risk of tumors in the lungs, brain, and liver. Similar events have not been observed in humans (445), where no difference in the incidence of any neoplasm has been observed with the use of ETV when compared to LMV.

ETV resistance is rare in NA-naïve patients and emerges slowly (450). ETV resistance is more common in the setting of prior LMV resistance, in spite of the increased 1-mg dose that is approved for this indication (see below).

Other Direct Acting Antiviral Drugs

Emtricitabine (FTC)

Emtricitabine (5′-fluorothiacytidine) is a fluorinated cytosine analogue that is structurally similar to LMV. It inhibits both HBV DNA polymerase and HIV reverse transcriptase. Forty-eight weeks of emtricitabine 200 mg daily reduced HBV DNA by 3 to 4 $\log_{10}$ copies/ml and significantly improved liver histology (451). However, despite virologic, biochemical, and histologic improvement, this study of 248 patients (63% HBeAg positive) failed to show any benefit in HBeAg seroconversion compared to placebo—12% in the two groups (451). Furthermore, FTC-resistance mutations in the YMDD motif (rtM204V) were detected in 13% of patients at 48 weeks, the mutations being cross-resistant for LMV. Preliminary clinical results suggest that rtM204V/I mutations occur in 19 to 37% of patients after 2 years of treatment (452).

FTC has been approved for the management of HIV. FTC monotherapy is unlikely to have an important role in the management of HBV, given the frequency of primary drug resistance and the issue of cross-resistance with LMV, but does hold promise in combination with tenofovir (TDF) for NA-naïve mono-infected patients and is used routinely in HIV/HBV coinfected patients.

Clevudine

Clevudine (2′-fluoro-5-methyl-beta-L-arabinofuranosyluracil) is a l-thymidine analogue with potent *in vitro* activity against HBV (453). It is a noncompetitive inhibitor of HBV Pol that inhibits viral (+)-(+) strand synthesis without incorporating itself into viral DNA. A unique property of clevudine is that it appears to suppress HBV DNA for a prolonged period after cessation of therapy (454). Clevudine, at a dose of 30 mg for 24 weeks (455, 456), reduced viral load to undetectable levels in 59% of HBeAg-positive and 92% of HBeAg-negative patients (455, 456), and statistically significant differences in viral suppression remained 24 weeks after cessation of therapy.

The safety of clevudine has been called into question, as several studies have shown an incidence of myopathy in 3.4 to 13.6% of patients (457–460). Given these safety

concerns, enrollment into planned larger phase III studies was terminated, and clevudine is currently marketed only in the Philippines.

Besifovir

Besifovir, formerly known as LB80380, is a novel nucleoside phosphonate analogue prodrug, similar in structure to ADV. It is, therefore, incorporated into the growing viral DNA strand causing termination and inhibition of replication. *In vitro* studies of efficacy demonstrated potent inhibition of HBV replication, leading to *in vivo* studies in the WHV model (461). Doses from 30 mg to 240 mg demonstrated significant reductions in HBV DNA without significant clinical or laboratory adverse effects (462). Besifovir also demonstrated efficacy in LMV-resistant infection (463). A phase IIb study, comparing besifovir at doses of 90 mg and 150 mg to ETV, 0.5 mg, showed equivalent degrees of viral DNA suppression between the three groups after 48 weeks of treatment (464). Increasing rates of viral suppression to approximately 80% undetectable have been seen after 2 years of maintenance therapy (465). Some concern has been raised over the decline in serum l-carnitine, seen in 94.1% of patients treated with besifovir, which required supplementation therapy. A similar effect was seen with high-dose ADV (466). Outcomes of a phase III study comparing besifovir 150 mg to tenofovir 300 mg for 48 weeks are awaited (NCT01937806).

Nucleos(t)ide Analogue Resistance

Drug resistance is important because it is associated with the loss of virological, biochemical, and eventually histological therapeutic gain. In the setting of advanced liver disease, resistance may lead to hepatitis flares, hepatic decompensation, and death (353). Drug resistance mutations also appear to be archived in the nuclear cccDNA template (467).

Primary resistance mutations are those that directly alter drug binding and thus confer drug resistance. These mutations commonly result in reduced viral replicative ability when compared to wild-type virus (468). Secondary resistance mutations arise in selected variants that have acquired primary resistance mutations and have the effect of causing a compensatory increase in viral replication levels.

Mutations Conferring Resistance

L-Nucleoside Analogue Resistance

Primary resistance to LMV and LdT has been mapped to mutations in the YMDD locus in the catalytic, or C, domain of HBV Pol (469). These occur primarily at rtM204I/V/S (Domain C), ± rtL180M (Domain B) (469), and rtA181T/V (Domain B) (470). Only the latter mutation confers cross-resistance to ADV or TDF (471). There are multiple secondary resistance mutations that can be selected for in LMV-resistant strains, some of which contribute to ETV resistance. Individually, these mutations do not significantly impact the efficacy of ETV (472).

Mutations that confer LMV resistance decrease *in vitro* sensitivity to LMV from at least 100-fold to greater than 1000-fold. The rtM204I substitution has been detected in isolation, but rtM204V and rtM204S are found only in association with other changes in the B or A domains (473). The major patterns of mutation are: 1) rtM204I, 2) rtL180M + rtM204V, 3) rtL180M + rtM204I, 4) rtV173L + rtL180M + rtM204V, and 5) rtL80V/I ± rtL180M + rtM204I. Genotype influences the dominance of a particular mutation sequence (474). The molecular mechanism of LMV resistance is steric hindrance caused by the branched side group of valine or isoleucine amino acids colliding with the oxathiolane ring of l-nucleosides in the dNTP binding site (475).

LMV resistance increases progressively during treatment at rates between 14% and 32% annually (Table 6) (476). The rate of resistance observed with LdT therapy is lower than for LMV; however, it is substantial and increases exponentially after the first year of therapy. Genotypic resistance was observed at 1 and 2 years of therapy in 4.4%/2.7% and 21.6%/8.6% in HBeAg-positive and -negative patients, respectively (230).

Acyclic Nucleoside Phosphonate Resistance

Resistance to ADV was initially associated with mutations in the B (rtA181T/V/S) and D (rtN236T) domains of the Pol enzyme (Fig 2B and Table 7) (477). These ADV-associated mutations in HBV Pol result in only a modest (three- to eightfold) increase in IC_{50} and are partially cross-resistant with TDF, probably because the molecular mechanism of resistance is similar in both, with indirect perturbation of the triphosphate binding site between the A and D domains (475). The rtN236T does not significantly affect sensitivity to LMV, LdT, or ETV, but it reduces the *in vitro* efficacy of TDF (477, 478). The rtA181T/V mutation is partially cross-resistant to LMV and LdT (471) and reduces sensitivity to TDF by approximately threefold (479). The rtA181S mutation appears to affect only ADV (480). Resistance to ADV occurs less frequently (around 2% after 2 years, 4% after 3 years, and 18% after 4 years) than resistance to l-nucleosides.

Resistance to TDF in patients with HIV-HBV coinfection is conferred by the rtA194T mutation in combination with rtL180M and rtM204V (481). *In vitro* the presence of rtA194T results in partial resistance to TDF but shows no cross-resistance with LdT or ETV. Additionally these virus strains had reduced replication efficiency compared to wild type, but this could be reversed by the introduction of either precore or BCP mutations (482). Clinical studies have not confirmed an *in vivo* effect of this mutation. Further *in vitro* studies have shown a 10-fold reduction in TDF sensitivity when the rtN236T and rtA181T/V mutations are found together.

D-Cyclopentane Resistance

Mutations in the viral polymerase associated with the emergence of ETV-resistance have been mapped to the B domain (rtI169T, rtS184G, and/or rtL180M), C domain (rtS202I and rtM204V), and E domain (rtM250V) (Fig 2B and Table 7). Thus, at least three mutations are required for resistance to develop. In the absence of the LMV mutations rtM204V/I and rtL180M, the rtM250V mutation causes a 10-fold increase in $IC_{50,}$ while mutations rtT184G+rtS202I have only modest effect (483–485). The molecular mechanism of resistance for the rtM250V change is exerted during RNA-directed DNA synthesis (485). The mutation reduces the dNTP-binding site by repositioning it, thus shifting the ETV binding pocket. The mechanism for the rtT184G/rtS202I combination is an allosteric change with altered geometry of the nucleotide binding pocket near the YMDD site (485). A newly recognized mutation at rtA186T confers primary ETV resistance in combination with LMV resistance mutations (486). The significance of this mutation will need confirmation in future studies.

Genotypic resistance to ETV is uncommon in NA treatment-naïve patients, seen in 1.2% of patients after 5 years of therapy (487). However, patients with LMV-resistance at commencement of ETV monotherapy develop

resistance rapidly (Table 6). This is because ETV resistance develops in a stepwise fashion, which means that a virus with YMDD mutations needs to develop fewer additional mutations to become resistant (472). Therefore ETV should not be used as monotherapy to treat LMV-resistant patients.

Importantly, ETV resistance-associated mutations are not cross-resistant with ADV or TDF.

Multidrug Resistance

Multidrug-resistant (MDR) HBV occurs in patients who received sequential NA monotherapy (478, 483,488–490). MDR strains have been shown to develop if an "add-on" therapeutic strategy to treat initial resistance to a single agent does not result in rapid and complete viral suppression (468).

The rtA181V/T mutation is a marker for multidrug resistance as it is responsible for reduced susceptibility to both the l-nucleosides and the acyclic phosphonate nucleosides (471, 491).

Detection of Drug Resistance

Viral load assay is the principal clinical tool used to detect and confirm the development of drug resistance while on treatment. When a significant rise in viral load is seen (>1 $\log_{10}$), patient compliance to therapy must be evaluated before ascribing the changes to resistance.

An analysis for genotypic resistance should be requested upon confirmation of viral breakthrough (defined above). Ideally, the nucleotide sequence of the HBV polymerase isolated from the patient during breakthrough should be compared with a sequence from a pretherapy sample from the same patient (492). In the absence of a paired sample, the sequence should be compared with published consensus sequences of the same HBV genotype (493). Determining which mutation/s are present will allow rational prescription of alternate therapy without cross-resistance (Table 7).

Management

Factors that increase the risk of development of resistance include high pretherapy serum HBV DNA, high ALT levels, and high body mass index (476, 494, 495). Patients previously exposed to NA therapy are also at higher risk for developing resistance (496).

Primary Nonresponse

Primary nonresponse is uncommon when using potent first-line agents, but is defined as a failure to achieve a 1 $\log_{10}$ reduction in viral load after 12 weeks of treatment (295). This has been shown to be related to pharmacogenomics factors and/or compliance issues in the case of ADV therapy, which is associated with the highest rate of primary nonresponse, occurring in 10 to 20% of patients (425). These patients should be quickly switched to either TDF or ETV therapy (497). Primary nonresponse to the other NA drugs is rare, and, in such situations, compliance must be carefully assessed (498). A compliant patient should then proceed to have genotypic resistance analysis (314).

Partial Virologic Response

A partial virological response is defined as detectable HBV DNA after 6 months of treatment using current sensitive assays (314). A viral load of >3 $\log_{10}$ copies/ml at this timepoint has been shown to predict resistance after 2 years of therapy with LdT or LMV (420, 496). The effect of ADV on viral kinetics is slower than the other agents, so this threshold at 48 weeks of therapy can be used to predict resistance (428). This threshold/resistance effect is seen with all NA used to treat CHB, but less so with TDF and ETV. It is again important to check compliance. If LMV, ADV, or LdT has been used, then switching to TDF or ETV can be done at week 24 in those who have had a partial response (468). A change in therapy is probably not necessary in those initially treated with TDF or ETV. In patients naïve to NA treatment and started on ETV, 21% had detectable HBV DNA at week 48. Despite this, 81% of this group proceeded to have a complete response by the end of the study at week 144 (499), and no resistance was seen. Similarly, studies in patients receiving TDF have shown continued virologic responses and no long-term resistance despite an up to 24% partial response rate at week 48 (431, 500).

Virologic Breakthrough

Virologic breakthrough is defined as a >1 $\log_{10}$ (10-fold) increase in serum HBV DNA from nadir, in two consecutive samples taken 1 month apart, in a compliant patient who had an initial virological response (295). Notably up to 40% of viral breakthrough is due to nonadherence to therapy (501), but in a patient adherent to therapy, virologic breakthrough is primarily due to viral resistance. Virologic breakthrough is usually followed by biochemical breakthrough but may occur months and sometimes years before biochemical breakthrough. Hence early detection and treatment is possible prior to the development of clinical complications. This is particularly important in the setting of advanced liver disease.

Ideally, resistance should be identified before ALT levels rise, and genotypic analysis should be performed in order to institute rescue therapy and avoid clinically significant consequences. The benefit of an early therapeutic adaptation has been shown in several studies (314, 502).

Summary

Current recommendations suggest (314, 468):

- Lamivudine resistance: switch to TDF (or add ADV if TDF not available).
- Telbivudine resistance: switch to or add TDF (or add ADV if TDF not available).
- Entecavir resistance: switch to or add TDF (or add ADV if TDF not available).
- Adefovir resistance: if naïve before ADV, switch to either ETV or TDF (ETV preferred if high viral load). If rtN236T mutation present, add ETV or switch to TDF/FTC. If rtA181V/T mutation present, add ETV or switch to either ETV/TDF or TDF/FTC.
- Multidrug resistance: consider TDF/ETV combination therapy.

L-Nucleoside Resistance

ADV is effective in the management of LMV resistant variants. However, despite early treatment response, following a switch to ADV monotherapy, rates of genotypic ADV resistance as high as 18% develop after 48 weeks (503). The optimal strategy for the use of ADV therapy in the setting of LMV-resistance is, therefore, to add-on ADV (504, 505). When used, ADV should be added on early (at the time of genotypic versus phenotypic resistance), when VL remains $<200{,}000$ IU/ml (10^6 copies/ml) to promote virological response (505, 506). Add-on therapy with ADV has also been successful when resistance to LdT monotherapy develops (507). This approach has a low rate of subsequent ADV resistance, occurring in 4% after 4 years of therapy (502).

Tenofovir (TDF) is also effective for the management of LMV-resistant HBV, resulting in a significant reduction in serum HBV DNA levels (508–511). It is more effective than ADV as primary salvage therapy and is also effective as secondary therapy following inadequate response to ADV salvage (508,512–514). A randomized study in patients with partial response to add-on ADV therapy after 6 months found complete virologic response in 96.4% of the TDF group versus 29.0% of those continuing LAM/ADV ($P < 0.001$) (515), confirming that TDF is the preferred agent in the event of LMV-resistance.

In studies with follow-up limited to 48 weeks, ETV monotherapy at a dose of 1 mg per day appeared to be effective for the management of LMV resistance (higher than the 0.5 mg per day recommended for NA-naïve patients) (516). However, the M204V/I LMV mutations are required as the first "hit" in the two-hit process producing ETV resistance (483). Additionally, commencing ETV therapy in patients with genotypic LMV-resistance significantly impairs the rate of complete viral response as compared to treatment naïve patients (HR 0.14 [CI: 0.04–0.58], $P = 0.007$) (517). Consequently, ETV resistance develops commonly with long-term therapy when used in patients with genotypic LMV-resistance, and ETV should not be used to treat genotypically proven l-nucleoside-resistant virus.

Adefovir Resistance

In vitro resistance profiles suggest that treatment options for ADV-resistance would include LMV, ETV, and TDF (Table 7) (518, 519). If used, LMV should be added on, especially in patients with a previous history of LMV-resistance, which has been reported to re-emerge rapidly following the re-introduction of LMV (488). Although the mutations are partially cross-resistant with TDF *in vitro*, the higher relative dose of TDF allows effective salvage therapy for ADV-resistance. In a randomized controlled trial of 105 ADV-treated patients with viral loads >1000 copies/ml despite 24 weeks of treatment (520), patients received either TDF monotherapy or combination therapy with TDF/FTC. At baseline, 10% of the cohort had genotypic ADV resistance with rtN23T and/or A181V/T, and 12% had LMV resistance. After 168 weeks of follow-up, >80% of patients in both arms had achieved DNA <400 copies/ml, irrespective of the presence of genotypic ADV resistance (520). A second study of TDF versus TDF/ETV in genotypic ADV resistance showed similar results. There was a trend towards a slower reduction in viral load seen only in those with dual rt181V/T and rtN236T mutations (521).

Entecavir Resistance

There are few large studies investigating NA therapy for ETV-resistant infection. In essence, ETV-resistance can only be managed with acyclic phosphonate drugs or interferon, as l-nucleoside resistance mutations are a prerequisite for ETV-resistance to develop.

The utility of add-on ADV therapy in patients with genotypic ETV resistance was shown in two small studies (522, 523), one of which enrolled 67 patients and added ADV 10 mg to l-nucleoside therapy or ETV. Treatment for 24 months was associated with complete viral suppression in 47.4% of patients, with no difference between LMV/LdT or ETV groups ($P = 0.23$) (522).

TDF has shown efficacy in ETV-resistant disease, both in combination with ETV and as monotherapy. A cohort of 57 heavily pretreated patients without complete virologic response included 24 (42%) who had ETV exposure (524). After a median of 6 months [IQR: 4.6–7 months] of treatment with ETV/TDF, 89% had undetectable HBV DNA. Another study randomized 90 patients, all with genotypically proven ETV resistance (525), to receive 48 weeks of either TDF monotherapy or combination therapy with TDF 300 mg/ETV 1 mg, both daily. At the end of treatment, 71% in the monotherapy group and 73% in the combination group had achieved a HBV DNA level <15 IU/ml ($P = 0.81$). A small study also showed good efficacy of TDF monotherapy in patients with suboptimal responses to ETV rescue (515). Thus, on balance, TDF/ETV rescue should be considered a last resort.

Prevention

Resistance will remain an important issue in the management of patients with CHB because long-term, and probably lifelong, therapy with NA will be required in the majority of patients. The use of high-potency, high-barrier-to-resistance first-line agents quickly reduce the viral load and quasi-species pool and reduce the risk of resistance. Adhering to best practice management and drug prescription can reduce the development of resistance. This includes avoidance of unnecessary drug use, continuous surveillance for resistance, prompt and appropriate rescue therapy if resistance emerges, and initial use of potent NA therapy with a high genetic barrier to resistance (TDF or ETV). Monitoring adherence to prescribed therapy, as well as on-treatment responses, should be performed using HBV DNA and ALT levels every 3 to 6 months for 2 years after commencing therapy. Polymerase gene sequencing upon the emergence of resistance should then be performed to determine the next therapeutic approach based on cross-resistance information.

Pol-HBsAg Mutations

The polymerase gene overlaps the envelope gene (Fig 9), and changes in the HBV Pol selected during antiviral resistance can therefore cause concomitant changes in the envelope gene. Thus, the major resistance mutations associated with NA failure also have the potential of altering the C-terminal region of HBsAg. For example, the rtM204V mutation associated with primary LMV and ETV resistance, results in a change at sI195M in the surface antigen, while the rtM204I change that is associated with LMV and LdT resistance is linked to three possible changes, sW196S, sW196L, or a termination codon (sW196*). To date, only one published study has examined the effect of the main LMV resistance mutations on the altered antigenicity of HBsAg (526). One of the common HBV variants that is selected during LMV treatment is rtV173L + rtL180M + rtM204V, which results in changes in the HBsAg at sE164D + sI195M. Approximately 20% of HIV-HBV coinfected individuals and 10% of mono-infected individuals carry this "triple Pol mutant" (527, 528). In binding assays, HBsAg-expressing triple-Pol mutants had reduced anti-HBs binding. This reduction was similar to the classical vaccine escape mutant, sG145R (44), and, subsequently, this virus successfully superinfected vaccinated chimpanzees (529).

The ADV-resistance mutation rtN236T does not affect the envelope gene and overlaps with the stop codon at the end of the envelope gene. The rtA181T mutation selected by ADV and/or LMV results in a stop-codon mutation at sW172stop (491). The ADV-resistance mutation at rtA181V results in a change at sL173F. HBV, with mutations that result in a stop codon in the envelope gene, requires the presence of a low percentage of wild type to enable viral assembly and release.

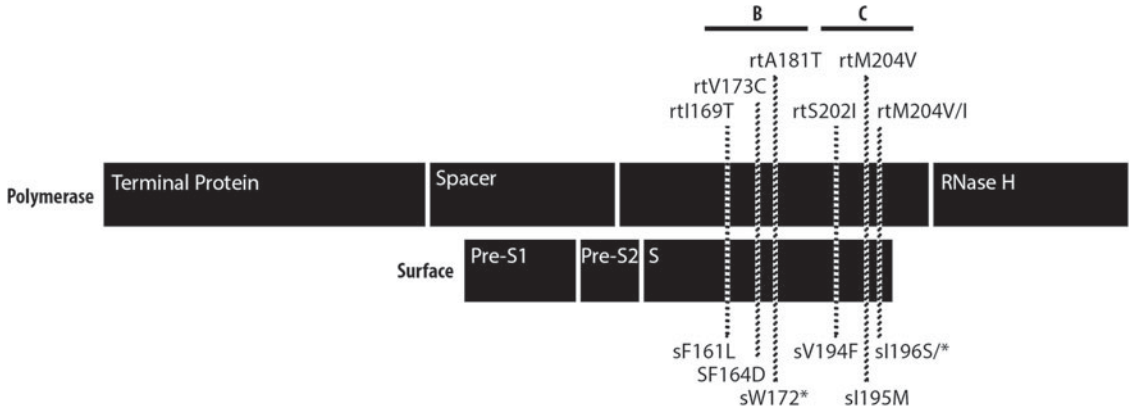


FIGURE 9 HBV DNA genome showing the overlapping open reading frames (ORFs) of POL and S, and, in particular, how the polymerase-envelope overlap can affect each other during the emergence of NA resistance.

The ETV resistance-associated changes at rtI169T, rtS184G, and rtS202I also affect HBsAg and result in changes at sF161L, sL/V176G, and sV194F. The rtM250V is located after the end of HBsAg, and thus has no effect on it. The sF161L is located within the "a" determinant, or major hydrophilic region, which includes amino acids 90 to 170 of the HBsAg (530). This region is a highly conformational epitope, characterized by multiple disulphide bonds formed from sets of cysteines at residues 107 to 138, 137 to 149, and 139 to 147 (530). Accordingly, changes to HBsAg that are driven by NA resistance, such as sF161L, need further investigation in order to determine the effect on envelope structure and the subsequent affinity of anti-HBs binding. Proof of this concept is seen with distal substitutions, such as sE164D, which has already been shown to significantly affect anti-HBs binding (526).

In summary, because of the overlap between the Pol and S genes, the selection of drug-resistant HBV has important clinical, diagnostic, and public health implications. The significance of these changes warrants further investigation to determine what effect they have on the natural history of drug-resistant HBV and its transmissibility in the HBV-vaccinated community.

Transmission of Resistant Strains

Transmission of infection with LMV-resistant HBV to an HIV patient undergoing LMV treatment as part of antiretroviral therapy has been reported (531). Another report notes baseline YMDD mutations in two treatment-naïve patients with HBV-HIV coinfection (532) and also in two cases with acute HBV infection (533). In addition, LMV-resistance mutations were found in a cohort of dialysis patients with occult HBV (534). Therefore, it is important to recognize that both primary- and secondary-resistance mutations may result in associated changes to the viral envelope that could lead to vaccine and immune escape (529), resulting in substantial public health relevance.

Stopping NA Therapy

The defined end point for HBeAg-positive infection is attaining sustained HBeAg seroconversion with undetectable HBV DNA. Cessation of NA therapy can be considered following at least 6 to 12 months of consolidation therapy after achieving this goal (230, 314, 361, 535). Patients will require ongoing monitoring as a proportion fail to sustain their serological or virological responses (362, 399, 400, 536).

The majority of HBeAg-negative patients will relapse after discontinuing NA therapy, and the role of quantitative HBsAg testing in predicting those HBeAg-negative patients likely to have a sustained response is unsettled. In one study of 53 HBeAg-negative patients, who stopped LMV after 12 to 76 months of treatment after having achieved suppression of HBV DNA, all patients who achieved a quantitative HBsAg decline to below 100 IU/ml and a >1 $\log_{10}$ decline continued to have suppressed HBV DNA at 12 months, whereas all who did not achieve these targets had virologic relapse (537). However, in a group of 184 patients, who had a minimum of 2 years of ETV therapy and at least 6 months of HBV DNA undetectability before cessation of therapy (538), the relapse rate was 91.4% after 48 weeks, which could not be predicted by baseline quantitative HBsAg or off-treatment quantitative HBsAg or viral load. Given these data, HBeAg-negative patients will require continuous NA therapy until HBsAg loss.

Immunomodulators

Interferon-α

Standard IFNα (sIFN) is effective in suppressing HBV replication and was the first treatment approved for chronic HBV infection in most countries (Tables 5A and 5B). Its use has been largely supplanted in Western clinical practice by pegylated-IFNα (pegIFN) due to the more convenient dosing schedule and equivalent efficacy. The attachment of polyethylene glycol to a protein (pegylation) reduces its rate of absorption following subcutaneous injection, reduces renal and cellular clearance, and decreases the immunogenicity of the protein. All of these effects enhance the half-life of the pegylated- versus the native-protein. This allows pegIFN to be administered once weekly, while maintaining a more sustained viral suppression between doses. One phase II clinical study has suggested that the efficacy of pegIFN is similar to, or slightly superior to, sIFN (539). The advantages of interferon, as opposed to NA, include the finite course of treatment and the durability of HBeAg seroconversion. Interferon has also been shown to enhance degradation of cccDNA by upregulating cytidine deaminases (540) and by inducing epigenetic silencing of the mini-chromosome (150). The disadvantages are the side-effect profile, the lack of durable viral suppression in HBeAg-negative infection, and the overall poor sustained response. Response to interferon therapy is influenced by genotype, and current management and treatment guidelines do not

recommend a role for genotype determination unless interferon therapy is being considered.

Standard Interferon-α

Standard interferon-α (sIFN) was one of the first therapies investigated for CHB. A meta-analysis of 15 randomized controlled trials using sIFN for the treatment of HBeAg-positive disease concluded that viral suppression, normalization of ALT, and HBeAg seroconversion (33% vs. 12%) were significantly more common compared to untreated controls if patients received sIFN for 3 to 6 months and were followed for 6 to 12 months. The loss of HBsAg occurred in 7.8% of sIFN-treated patients compared to 1.8% controls ($P = 0.001$). In long-term follow-up studies the durability of sIFN-induced seroconversion has been reported to be 80 to 90% at 4 to 8 years (356–358, 360, 541–544).

Clinical outcome studies comparing responders and nonresponders have found that HBeAg seroconversion predicted better long-term survival and survival free of hepatic decompensation (357, 358, 360, 364, 545). Only one prospective randomized controlled trial has compared clinical outcome following sIFN with placebo (356). This Taiwanese study found treated patients to have a lower incidence of HCC (1.5% vs. 12%, $P = 0.04$) and improved survival (98% vs. 57%, $P = 0.02$) after a median 8-year follow-up.

sIFN also has an antiviral effect in HBeAg-negative CHB. Four randomized controlled trials demonstrated an end-of-treatment response in 38 to 90% of patients compared to only 0 to 37% in controls (546–549). In comparison to HBeAg-positive CHB, relapse posttherapy is frequent, with sustained response rates of only 15 to 30% (550, 551). Late relapse has been noted out to 5 years.

The use of sIFN in the setting of cirrhosis is limited by the risk of hepatitis flare, which may precipitate hepatic decompensation. Approximately 20 to 40% of HBeAg-positive patients develop a flare of their ALT during treatment. In compensated cirrhosis the risk appears to be small, and less than 1% of cirrhotic patients included in HBeAg-positive cohorts developed hepatic decompensation (552, 553). Treatment response was comparable to precirrhotic patients. However, sIFN offers little virologic benefit once liver disease progresses to Childs class B or C and is complicated by significant toxicity and exacerbations of liver disease, even at low dose (554, 555). IFN is therefore contra-indicated in this setting, and NA therapy provides a safe and effective alternative.

sIFN therapy is associated with multiple adverse effects (see below).

Pegylated Interferon-α (pegIFN)

pegIFN is useful for both HBeAg-positive and -negative CHB. In the largest phase III trial of over HBeAg-positive 800 patients, 48 weeks of pegIFNα-2a at a dose of 180 μg/week was compared to the combination of pegylated IFNα-2a plus LMV 100 mg daily or LMV monotherapy (364). At the end of treatment, combination therapy achieved the most profound viral suppression but HBeAg seroconversion was similar (27%, 24%, and 20%, respectively). The HBeAg seroconversion rate was, however, significantly higher in the two groups that received pegIFN therapy at 24 weeks posttreatment being 32% and 27% vs. 19% ($P < 0.02$). Sixteen patients receiving pegIFN (1.5%) experienced HBsAg seroconversion as compared with no patients in the LMV monotherapy group ($P = 0.001$). The combination did not offer additional benefit. Similar results have been obtained in other studies using varying regimens of pegylated IFNα-2b (556, 557). The standard regimen of 180 μg/week of pegylated IFN-2a for 48 weeks has better efficacy than either lower dose, or shorter duration, regimens (558).

A number of baseline factors predict response to pegIFN in HBeAg-positive patients. HBeAg seroconversion is more likely in the setting of an ALT >2 x ULN, low HBV DNA ($< \log_{10}9$ IU/ml), female gender, older age, and naïve to previous interferon-based therapy (559). Genotype also appears to be an important factor: genotype-A disease requires either elevated ALT or low DNA to predict SVR, whereas genotype D disease has a poor response regardless. Genotype B and C require both high ALT and low DNA to predict good response. This carries over to HBsAg seroconversion, which is more likely in the setting of genotype A disease, occurring in 14% after 52 weeks of therapy versus 2% in genotype D (560). These factors are associated with good long-term responses, with >80% of patients maintaining HBeAg seroconversion out to 3 to 5 years (561, 562).

Fewer studies exist that examine the use of pegIFN for the treatment of HBeAg-negative CHB. The pivotal study used a design similar to the HBeAg-positive phase III trial above and compared pegIFN alone, or in combination with LMV, to LMV monotherapy (563). Sustained viral response, defined as undetectable HBV DNA (<400 copies/ml), was 19% and 20% in the groups that received pegIFN, versus 7% in the LMV monotherapy group ($P < 0.001$ for both comparisons). After 3 years, however, only 25% of patients achieving SVR maintained it (564). Predictive factors for SVR in HBeAg-negative infection have been found to be similar to HBeAg-positive patients (565).

HBeAg-negative genotype D patients are perhaps one of the most difficult groups to treat. A prospective, randomized study compared 180 μg/week of pegIFN for 48 weeks to a group treated with 135 μg/week of pegIFN for an additional 48 weeks (566). A higher proportion of the extended therapy group (28.8% vs. 11.8%, $P = 0.03$) had a virologic response, defined as HBV DNA <2000 IU/ml 48 weeks after treatment cessation, so that the prolonged regimen is standard of care for this patient population.

The limitation of all studies that have compared pegIFN to LMV is that both LMV and pegIFN were stopped simultaneously (in HBeAg-positive patients before the majority had achieved HBeAg seroconversion) (567). Primary end points were defined at 24 weeks posttreatment. In clinical practice, LMV would be continued until a clinical end point was achieved; it has been shown that virologic relapse is almost universal otherwise. In a study of pegIFN in 16 patients with LMV-resistant YMDD mutations, only two patients seroconverted and achieved sustained virologic suppression and biochemical normalization (567).

Like sIFN, pegIFN should be used with caution in patients with advanced liver disease, as a treatment-induced flare might precipitate hepatic decompensation. Although there are no data specifically addressing the use of pegIFN in cirrhotic patients, it is reasonable to extrapolate from the experience with sIFN. Thus, only patients with compensated cirrhosis should be considered for treatment. Further, in the two phase III trials approximately 15%/25% of the HBeAg-positive and -negative cohorts had advanced fibrosis/cirrhosis on liver biopsy, respectively, and no instances of hepatic decompensation were recorded (568). pegIFN should not be used for patients with Child-Pugh class B or C cirrhosis.

Both IFN preparations have similar side-effect profiles. Common symptoms include an initial flu-like illness, anorexia, weight loss, fatigue, mild alopecia, and skin rashes.

Significant neurocognitive effects are seen and commonly include emotional lability and poor concentration. Additionally, anxiety, irritability, depression, and even suicidal tendencies have been described. IFN is myelosuppressive, although profound neutropenia or thrombocytopenia is uncommon. Regular monitoring of blood counts is required during therapy. Autoimmune disease has been described, most commonly hyper- or hypothyroidism. Retinal changes and impaired vision occur rarely. A hepatitis flare is precipitated in 30 to 40% of patients undergoing IFN therapy for CHB. Although this is considered to be a marker of treatment response, indicating an increased likelihood of HBeAg seroconversion, it may precipitate hepatic decompensation, especially in the setting of cirrhosis.

On-Treatment Predictors and Response-Guided Therapy

Given the side effects and cost of pegIFN-based regimens, interest has been directed towards delineating stopping rules for those patients who are unlikely to achieve a SVR. Much of this research has investigated the utility of quantitative HBsAg levels. Pretreatment quantitative HBsAg levels are not clinically useful (569, 570). However, on-treatment HBsAg levels at weeks 12/24 can be used to guide treatment decision-making. In HBeAg-positive patients infected with genotype B or C, week 12 HBsAg levels >20,000 IU/ml have a NPV of 92 to 98% for SVR. In genotype A or D patients, the absence of a quantitative HBsAg decline at week 12 has a negative predictive value of 97 to 100%. At week 24, HBsAg levels >20,000 IU/ml have a NPV 96 to 100%, irrespective of genotype (571). These thresholds therefore have utility as stopping rules, now acknowledged in clinical guidelines (314).

There is also a week-12 HBsAg futility rule for HBeAg-negative patients with genotype A or D CHB treated with pegIFN. At week 12, the combination of failure to achieve any quantitative HBsAg decline plus an HBV DNA reduction <2 $\log_{10}$ IU/ml has a NPV of 95 to 100% (572, 573). Genotypes A to D were represented, although genotype D comprised about 55% of the population. This approach has since been found to be cost-effective in European populations (574). Validation in Asian populations is needed.

The utility of quantitative HBsAg in predicting longer term treatment response has been examined in HBeAg-negative patients in two studies. The first followed patients at 3 years and showed that an end-of-treatment quantitative HBsAg <10 IU/ml predicted a 52% chance of HBsAg seroconversion versus a 2% chance if this threshold was not reached (575). The second study demonstrated the importance of on-treatment quantitative HBsAg kinetics in predicting HBsAg seroconversion out to 5 years posttreatment. This was achieved by 23% of patients in whom quantitative HBsAg declined by at least 10% by week 12, compared to 7.5% in those whose levels declined by less than 10% (576).

Quantitative HBeAg levels have been less well studied as a prediction tool and would only be useful for HBeAg-positive infection. A *post hoc* analysis determined that an HBeAg level of >100 PEIU/ml at week 24 had a NPV of 96% for HBeAg seroconversion 6 months posttreatment (319).

Thymosin-α-1

Thymosin-α-1 (Tα-1) is a synthetic thymic extract. Thymosin derivatives regulate multiple aspects of T-cell function. *In vitro* studies have shown that Tα-1 can influence T-cell production and maturation, stimulate production of T-helper cell 1 (Th1) cytokines, such as interferon-γ (IFN-γ) and interleukin-2 (IL-2), and activate natural killer cell-mediated cytotoxicity (568,577–586).

Tα-1 is approved for the treatment of HBV in several countries, mainly in Asia. Treatment is usually well tolerated but antiviral efficacy remains controversial with a number of smaller clinical trials finding conflicting results (582, 584–586). A meta-analysis published in 2001, that included a total of 353 patients from five controlled trials, concluded that patients treated with Tα-1 were significantly more likely than controls to have a virologic response, defined as loss of HBV DNA and HBeAg (578). The maximal rate of response was not seen until 12 months after discontinuing therapy (odds ratio 2.67, 95% CI 1.25–5.68). Another meta-analysis examined 583 HBeAg-positive patients across eight trials randomized to either LMV alone or combined LMV/Tα-1 therapy. Superiority of combination therapy was shown for HBeAg seroconversion (45.1% vs. 15.2%, $P < 0.00001$) (587). Tα-1 offers the benefit of improved tolerability when compared to IFN therapy (582). Data on clinical efficacy are promising but confirmation in larger prospective randomized trials is required.

Combination Therapy

De novo combination of two NA drugs in treatment-naïve patients has not, overall, been shown to improve responses or outcomes. In a randomized study of HBeAg-positive patients after 2 years of treatment, no greater viral suppression occurred with LMV/ADV over LMV monotherapy, although the incidence of new M204V/I resistance mutations was reduced (588). The combination of LMV/LdT also showed equivalent viral suppression when compared to LMV monotherapy after 52 weeks, and, concerningly, a trend towards increased LdT resistance was seen in the combination group (417). This confirms that drugs within the same class should not be used concurrently. In a study comparing the combination of TDF/ETV to ETV alone, there was no difference in the primary outcome measure of undetectable HBV DNA after 100 weeks of treatment (83.2% vs. 76.4% for ETV monotherapy, $P = 0.088$) (589), although a *post hoc* analysis showed that the subset of patients, who were HBeAg-positive and with viral loads >7 $\log_{10}$ copies/ml, had a higher rate of complete HBV DNA suppression with combination therapy. This finding needs confirmation in prospective trials.

Because a significant immune dysfunction occurs in the presence of high levels of viral antigen, there is a theoretical basis for the use of NA to reduce antigen levels, to restore immune function, and to optimize the immunomodulatory effect of subsequent pegIFN therapy. Multiple studies have compared combined pegIFN-NA therapy using either concomitant or staggered treatment schedules (590). Three large pivotal trials using pegIFN and LMV established the superiority of pegIFN over LMV in achieving HBeAg seroconversion after 1 year of treatment, but combination therapy with both agents was not shown to improve sustained response over pegIFN monotherapy, despite greater on-treatment viral suppression (364, 556, 557). A small study of pegIFN and LdT showed increased HBeAg seroconversion, but patients suffered a high rate of peripheral neuropathy (591). More promise has been shown using add-on pegIFN therapy than to viral suppression achieved with ETV. The first study started all patients on ETV, 0.5 mg, and randomized them to either pegIFN add-on therapy from week 24 to 48 or to continued ETV monotherapy (592). Although the primary outcome of HBeAg loss plus HBV

DNA <200 IU/ml at 48 weeks was equivalent between the two groups, add-on therapy resulted in a higher HBeAg loss at 96 weeks (26% vs. 13%, $P = 0.036$). The second study selected patients treated with ETV who had achieved HBeAg <100 PEIU/ml and HBV DNA <1000 copies/ml. These patients were randomized to continued ETV therapy or to switch to 48 weeks of pegIFN. At end-of-treatment, those who switched had higher HBeAg seroconversion rates (14.9% vs. 6.1%, $P = 0.047$) (593). Additionally, 8.5% of patients who switched also lost HBsAg.

In HBeAg-negative patients, the registrational trial showed no difference in virologic response or clinical outcomes between the pegIFN/LMV arm and the pegIFN monotherapy arm (563). Subsequent studies using either LMV or ADV have confirmed these findings (594, 595).

One large study including both HBeAg-positive and -negative patients, randomized them to one of three arms: TDF monotherapy, pegIFN monotherapy, or TDF/pegIFN combination therapy (596). After 48 weeks of treatment, 7.3% of patients in the combination arm achieved HBsAg loss versus 2.8% with pegIFN monotherapy and 0% with TDF monotherapy.

In summary, the weight of evidence for combination therapy has not definitively shown improved outcomes. Adding on or switching therapies in those with suppressed viral replication shows promise but needs replication in future studies, and data on individualizing therapeutic regimens for patients who will be most likely to benefit from such strategies requires further investigation.

Novel Therapeutic Strategies

1) *Toll-Like Receptor Ligands*. In the transgenic HBV mouse model, TLR3, 4, 5, 7, and 9 ligands exerted an indirect antiviral effect via induction of type 1 IFN (122). Using the hydrodynamic-injection mouse model of HBV infection, TLR4 ligands have shown an antiviral effect (597). The TLR2 ligand Pam-2-Cys has been shown to have direct antiviral efficacy in a cell-culture model of HBV replication (121). An oral agonist of TLR7, known as GS-9620, showed promise in preclinical studies of HBV-infected chimpanzees (598). Viral load declined by a mean of 2.2 logs, and effects persisted for months after cessation of therapy. Use in the woodchuck model showed similar sustained responses after a short course of therapy (599). Despite these positive results, two phase Ib studies of GS-9620 demonstrated no change in quantitative HBsAg levels or HBV DNA (600). Although a transient, dose-dependent induction in peripheral interferon-stimulated gene (ISG)-15 expression was seen, no corresponding increase in IFN α was detected. Seventy-five percent of patients were HBeAg-negative. The results of further studies in different populations are awaited (NCT02166047 and NCT01590654).
2) *Liver-Specific Drug Delivery*. Several experimental approaches have been taken: conjugation of antiviral agents to ligands that are selectively taken up by the liver, creation of prodrugs that require activation in the liver (601, 602), and use of a specific monoclonal antibody to recognize amino acids 183 to 191 of the envelope protein, as presented by the HLA-A201 MHC class I molecule. The monoclonal antibody therefore mimicked the specificity of the T cell receptor (603). This delivery system was used *in vitro* to selectively deliver IFN specifically to cells by conjugating the antibody to an interferon molecule (604). This may provide a future delivery platform for targeting HBV infected cells.
3) *RNA Interference*. *In vitro* and animal studies have suggested a potential therapeutic role for synthesized exogenous siRNA molecules targeted against HBV RNA transcripts (605–609). A delivery platform consisting of a hepatocyte-targeted endosome delivery system combined with a cholesterol-conjugated siRNA molecule targeted against HBV, known as ARC-520, has been developed and tested in mouse models (610). Subsequent phase I and IIb trials have been reported, showing a good safety profile and sustained quantitative HBsAg reductions in ETV-treated HBeAg-negative patients (611). Another phase II trial is ongoing, testing ARC-520 in combination with either TDF or ETV, in patients in the immune elimination phase (NCT02349126).
4) *Immunotherapy*. Therapeutic vaccine approaches have included a highly immunogenic pre-S1/pre-S2/S vaccine (339), DNA vaccines (612, 613), and T cell vaccines (614–616). Therapeutic vaccination has also been combined with PD-1 blockade (617) (see below) and NA therapy (618) to enhance the efficacy of the immunization in the functionally exhausted immune environment characterized by CHB infection. To date, despite promising results in animal models, this has not translated to durable responses in human infection (619–622). The *ex vivo* culture and priming of human dendritic cells with viral antigens, followed by autologous transfusion, is being explored as a form of immunotherapy (623–626).
5) *Reversal of Exhaustion*. As discussed previously, T cells of chronic HBV-infected patients exhibit immune exhaustion characterized by an increase in inhibitory surface molecules, such as PD-1. Experiments using CD8+ T cells from patients with CHB have revealed that *in vitro* blockade of PD-1 can restore HBV antigen-specific responses with regards to both proliferative capacity and cytokine production (627). *In vivo* blockade of PD-1 in the woodchuck model has shown similar results, with restoration of PBMC responses to HBcAg (617). Similarly, blockade of other mediators of immune paresis, such as Tim-3 and miR-146a, enhances T cell responses (200) (190).
6) *T-Cell Therapy*. Adoptive transfer of activated T-cell subsets, directed against HBV, into CHB patients may control replication (628). Other investigators have designed chimeric T-cell receptors directed against HBV surface proteins present on HBV-infected cells and used them to graft primary human T cells with antibody-like specificity via a retroviral transfection technique (629). The receptors were composed of a single-chain antibody fragment directed against HBV S or L protein fused to intracellular signaling domains of CD3ζ and the costimulatory CD28 molecule. This method was shown to provide T cells with the ability to recognize HBsAg-expressing hepatocytes, lyse these cells, and liberate cytokines (629). Further studies of this chimeric antigen receptor therapy showed *in vivo* efficacy of viral suppression in the mouse model (630).
7) *Entry Inhibition*. These molecules would be useful in the settings of postexposure prophylaxis, organ transplantation, reactivation after immunosuppression, and

perinatal infection (51). Initial studies showed that acetylated peptides, derived from the large envelope protein, could prevent HBV infection in animal models (631). A specific molecule called Myrcludex-B has been developed, which is a synthetic lipopeptide derived from the pre-S1 domain of the HBV envelope protein (632). Preclinical studies in immunodeficient mice has demonstrated that Myrcludex-B not only prevents intrahepatic viral spread but also hinders the amplification of the cccDNA pool in initially infected hepatocytes (77). Early reports of a phase IIa clinical study in patients showed a dose-dependent decline in serum HBV DNA levels (633).

8) *Nucleocapsid Inhibition*. A high-throughput screening method identified isothiafludine, a novel molecule that inhibited the pgRNA encapsidation step of the HBV replication cycle (634). This inhibition occurred via blockade of the interaction between pgRNA and HBcAg and led to an accumulation of replication-incompetent capsids. Studies, both *in vitro* and using the duck HBV model of infection, found a dose-dependent reduction in HBV DNA replication with isothiafludine therapy (634). As both wild-type and drug-resistant strains were equally affected, further development of nucleocapsid inhibitors may lead to future therapies.
9) *cccDNA Inhibitors*. Clearly, these molecules would be key to eliminating the long-lived cccDNA pool responsible for occult and persistent infection. Lymphotoxin-β receptor-specific activating antibodies have been shown to induce cccDNA degradation in a similar way to IFN. This occurs via induction of cytidine deaminases (specifically APOBEC3B) via IFN-independent pathways. This induction has also been shown to be safe, as well as more potent and durable than the response to IFN (540). The possibility of using epigenetic modification of cccDNA to reduce its transcription (635, 636) or harnessing specific endonucleases to target and cleave cccDNA without affecting host DNA (637–640) are other strategies. Other therapeutic strategies are summarized in Table 8.

LIVER TRANSPLANTATION FOR CHB

Liver transplantation for hepatitis B infection in the 1980s was associated with rapid viral recurrence, despite prophylactic HBIG, with resultant poor outcomes (641, 642). With the advent of the NA, in combination with high dose HBIG to prevent graft reinfection, transplantation with viral control became possible. However, HBIG is expensive, not readily available worldwide, and disease progression associated with drug resistance was common. Low-dose HBIG plus LMV provides safe and effective prophylaxis at significantly lower cost (643). The use of potent single agents has prevented reinfection and resistance, with equivalent outcomes irrespective of HBIG use (644, 645). NA therapy reduces the risk of clinical progression in patients with advanced fibrosis or cirrhosis (353, 413). Effective viral suppression has also allowed the salvage of many patients with decompensated cirrhosis, obviating or delaying the need for liver transplant (230, 646). Liver transplant outcomes for CHB are now equivalent to other liver diseases with a 5-year survival of 75% (647). Viral control can result in a dramatic improvement in liver function in the pretransplant patient, such that transplantation for decompensation is no longer required for some patients.

CONCLUSION

Despite vast gains in our knowledge of the natural history and pathogenesis of this important human pathogen, it remains a significant global cause of morbidity and mortality. The increasing number of countries with national immunization programs and the use of newborn active-passive immunization will greatly reduce the number of incident cases in the coming decades. Unfortunately, the developing world is lagging behind, and new, more cost-effective methods of

TABLE 8 Summary of potential novel therapeutic targets in chronic HBV, their mechanism of action, and current phase of study

Immune Modulation		
Mechanism	Examples	Phase/s of Study
Therapeutic vaccine	ABX-203, GS-4774, INO-1800	Phase I–III
TLR7 Agonist	GS-9620	Phase II
Immunotherapeutic	TG1050	Phase I
Immune modulator	CYT107	Phase I/IIa
Non-Nucleoside Antivirals		
Mechanism	Examples	Phase/s of Study
Entry inhibitor	Myrcludex B	Phase II
Capsid inhibitor	NVR-1221, Bay 41-4109	Phase I-IIa
RNAi gene silencer	ALN-HBV, ARC-520	Preclinical—Phase II/III
HBsAg release inhibitor	REP-9AC	Phase II
SMAC mimetic	Birinapant	Phase I/IIa
cccDNA inhibitor	BSBI-25	Preclinical
Small molecule nucleic acid hybrid	SB9200	Phase II
HBsAg inhibitor	TKM-HBV	Phase I
Cyclophilin inhibitor	NVPO18, CPI-432-32	Preclinical

(Adapted with permission from the Hepatitis B Foundation Drug Watch [651]).

preventing transmission are required. In order to address this deficit, WHO is developing a Global Health Sector Strategy on Viral Hepatitis (2016–2021), which will provide a platform of realistic goals to improve access to screening and treatment (648). In those who are identified as being chronically infected, the risk of complications of end-stage liver disease has been virtually negated in Western practice with the introduction of effective suppressive therapies and monitoring strategies. Despite this advance, a lasting cure for CHB remains elusive, with almost all patients being relegated to lifelong treatment. Future work is now focusing on the development of strategies and novel therapeutics to enhance functional and/or absolute cure, although a true solution is likely many years away from an introduction to routine clinical practice.

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Human Lymphotropic Viruses: HTLV-1 and HTLV-2

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Human T-cell lymphotropic virus types 1 and 2 (HTLV-1 and HTLV-2) and the more recently recognized HTLV-3 and HTLV-4 are human retroviruses of the genus *Deltaretrovirus* originally derived from closely related simian viruses. Proviral DNA is integrated in the host genome and propagated by lymphocytic division with only a minimal production of infectious virus. A small viral genome encodes several structural and regulatory proteins that in concert with the host cellular immune response control the burden of infection, as measured by the proportion of lymphocytes harboring HTLV proviral DNA. Most chronically infected humans are asymptomatic, but 2% to 4% of HTLV-1 carriers develop a mature T-cell malignancy called adult T-cell lymphoma (ATL). HTLV-2 infection does not cause malignant disease. Another 1% to 2% of HTLV-1 and HTLV-2 carriers develop a spinal cord disease known as HTLV- associated myelopathy (HAM) characterized by a progressive weakness and spasticity of the lower extremities as well as a hyperactive bladder. Various inflammatory conditions have been associated with infection, and long-term mortality may be increased. HTLV-1 and HTLV-2 are transmitted from mother to child via breast-feeding, by sexual intercourse, and parenterally by the infusion of infected blood or injection drug use. From its presumed origins in Central Africa and Melanesia, HTLV-1 has spread globally along with human migrations and the historical slave trade. HTLV-2, endemic in Amerindian and African pygmy populations, has had a more limited geographic distribution except for hyperendemic spread among injection drug users (IDUs) in the United States and Europe.

VIROLOGY

Classification

Within the taxa of DNA and RNA reverse-transcribing viruses, the HTLVs, along with bovine leukemia virus, are classified within the genus *Deltaretrovirus* in the family *Retroviridae* (1). HTLV-1 and HTLV-2 are RNA viruses that contain a diploid genome that replicates through a double-stranded DNA intermediary able to integrate into the host T-lymphocyte genome as a provirus. The integration process is essential to the ability of this class of virus to cause lifelong infection, evade immune clearance, and produce diseases of long latency such as leukemia/lymphoma and the inflammatory neurologic disease HAM. The high degree of sequence stability of the viral genome—despite hundreds of thousands of years of infecting humans—arose because HTLV-1 favors viral expansion through the proliferation of proviral DNA-harboring cells rather than the infection of new cells by cell-free virions like HIV (2). Therefore, the replicative machinery of the cell, rather than the error-prone viral reverse transcriptase, is responsible for maintaining viral genomic stability. The phylogeny of the HTLVs has become more complex as systematic studies of nonhuman primates and humans throughout the world have been carried out. A close relationship exists between the human and simian versions of these viruses. Figure 1 shows the phylogenetic relationship between the simian and human versions of the primate T-lymphotropic viruses. HTLV-3 is genetically equidistant (approximately 62% homology) from HTLV-1 and HTLV-2, which in turn are approximately 60% homologous (3, 4). HTLV-4 has only been reported in one individual from West Central Africa (5).

No serotypes for either HTLV-1 or HTLV-2 correspond to these genetically defined types. Serologically, there is significant cross-reactivity between HTLV-1 and HTLV-2, reflecting their approximate 65% sequence homology. There are limited data on seroreactivity for HTLV-3. The strongest antibody response to the major antigens of HTLV-1 and HTLV-2 occurs to the major capsid antigen p24, whereas the matrix protein p19 has approximately 50% homology and is less cross-reactive. No epidemiological data suggest that disease incidence or subtype is impacted by viral subtype for either HTLV-1 or HTLV-2.

Composition of the Virus

The HTLV-1 virion is approximately 100 nanometers in diameter, with a thin electron-dense outer envelope and an electron-dense, roughly spherical core. The total provirus genome consists of approximately 9,000 nucleotides, with identical sequences termed long terminal repeats (LTRs) at the 5′and 3′ ends of the genome, which in the case of 5′ LTR contain regulatory elements called Tax-responsive elements that control virus expression and virion production (Fig. 2). HTLV-3 has only two Tax-responsive elements in its LTR, while both HTLV-1 and HTLV-2 have three (6).

The major structural and regulatory proteins of HTLV-1 are summarized in Table 1. HTLV-1 shares with other

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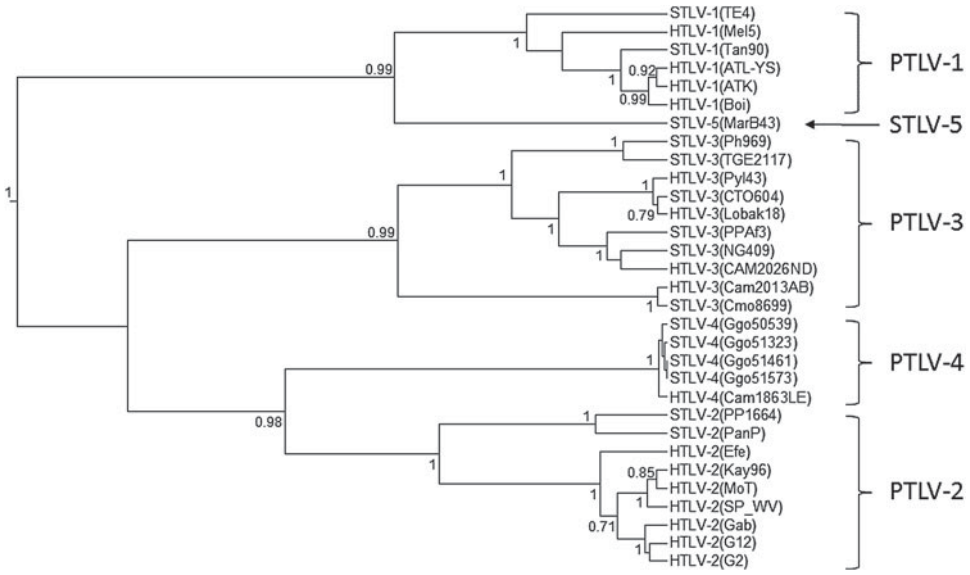


FIGURE 1 Phylogenetic relationships of HTLV-1, HTLV-2, HTLV-3, and HTLV-4 inferred using Bayesian analysis and a relaxed molecular clock. Topology inferred using the first and second codon positions of concatenated *gag*, *pol*, and *env* sequences (3,490-bp). Posterior probabilities greater than 0.7 are provided at branch nodes. (We are grateful to Dr. William Switzer for his development of and permission to use this figure.)

replication-competent retroviruses the three main genomic regions of *gag* (group-specific antigen), *pol* (protease/polymerase/integrase), and *env* (envelope). Production of the Gag proteins occurs as a result of the translation of the full-length mRNA, which yields a large precursor polypeptide that is subsequently cleaved by the virally encoded protease. For the Pol proteins, production depends on translation made possible when the stop codon of the *gag* gene is bypassed, leading to a large polypeptide including Gag- and Pol-related proteins, which are subsequently cleaved into functional proteins by the viral protease. Production of the Env surface and transmembrane proteins involves translation of spliced mRNA, which results in an envelope precursor that is cleaved into the subunits. The precursor proteins have characteristic molecular weights (MWs) that can be detected immunologically by Western blot analysis (Table 1). The Gag proteins function as structural proteins of the matrix, capsid, and nucleocapsid. The *pol* gene encodes several enzymes: a protease that cleaves Gag and Gag-Pol polypeptides, a reverse transcriptase that generates a double-stranded DNA from the RNA genome, and an integrase that integrates viral DNA into the host cell chromosomes. The *env* gene encodes the major components: the surface glycoprotein (gp46; MW, 46,000) and the transmembrane glycoprotein (gp21; MW, 21,000).

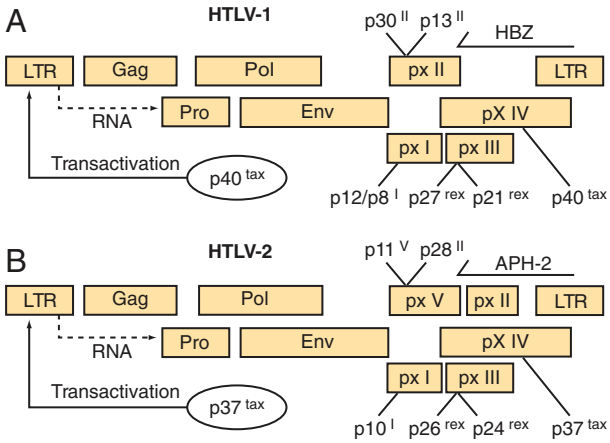


FIGURE 2 Genomic structures of HTLV-1 and HTLV-2. A. HTLV-1. B. HTLV-2. LTR, long terminal repeat; *gag*, group-specific antigen whose products form the skeleton of the virion (matrix, capsid, nucleocapsid, nucleic acid binding protein); *pro*, gene for protease; *pol*, gene for reverse transcriptase and integrase; *env*, envelope gene; *rex*, viral regulatory gene involved in promoting genomic RNA production; *tax*, transactivator gene; HBZ, antisense transcribed HTLV-I basic zipper gene involved in cell proliferation; and APH-2, antisense transcribed Antisense Protein of HTLV-2 gene involved in transcription regulation.

Unlike other vertebrate leukemia viruses, the deltaretroviruses have an additional region called pX that contains four open reading frames (ORFs). pX ORFs III and IV code for two transcription-regulatory proteins, the Tax and Rex proteins, whose functions are involved in the regulation of virus expression. pX ORFs I and II encode other regulatory genes whose functions are still a matter of research (7). Tax is responsible for the enhanced transcription of viral and cellular gene products and is essential for the transformation of human T lymphocytes (8); Rex (the regulator of expression of virion proteins for HTLV) promotes the export of non-spliced or singly spliced viral mRNA from the nucleus (9). As shown in Fig. 2, two overlapping reading frames are involved in the expression of both of these gene products, translated from a doubly spliced mRNA employing the initiation codon from *env* and the remaining sequences from the pX region. pX ORF I, also produced by this double-splicing mechanism, codes for a hydrophobic 12-kilodalton protein, $p12^{I}$; in some HTLV-1 strains, p12 is cleaved, resulting in p8, which plays a role in viral transmission (10). pX ORF II results in the production of two nuclear proteins, $p13^{II}$ and $p30^{II}$ (11). Antisense RNA from the negative

TABLE 1 Major structural and regulatory proteins of human T-lymphotropic virus type 1

Viral gene or region	Gene product (Protein size [kDa])	Function
5′ LTR		Regulation of viral gene expression and regulation of viral expression
5′ LTR & 3′ LTR		Integration points for provirus into host genome
hbz	HBZ	*hbz* mRNA is found in ATL tumor tissue and is responsible for lymphocyte proliferation; HBZ RNA is also involved in cell cycle regulation as it increases G1/S transition; HBZ protein prevents/suppresses Tax-mediated viral transcription, suppresses the nuclear factor *k*B (NF-*k*B) pathway, impairs the production of Th1 cytokines, and enhances TGF-β expression
gag	p15	Nucleocapsid (NC) is a small basic protein found in the virion in association with genomic RNA
gag	p19	Matrix (MA) protein is myristylated and anchored in the plasma membrane
gag	p24	Capsid (CA) protein forms the major internal structural feature of the core shell of the virion
gag	p53	Precursor protein for other Gag proteins
pro	Protease	Cleaves Gag precursor into CS, MA, NC, Pol precursor in RT and IN proteins
pol	Integrase	Integrates viral DNA into the host-cell genome
pol	Reverse transcriptase (95 kDa)	Reverse transcriptase generates a double-stranded DNA from the single- strand RNA genome
env	gp21 and gp46	Envelope transmembrane and surface glycoproteins
pX	Tax (p40)	Transactivator, enhances transcription of viral promoter and alters transcription from cellular gene promoters; recruits cyclic AMP response element binding proteins/activating transcription factors (CREB/ATF); activates signaling pathways including NF-*k*B, activator protein-1 (AP-1), serum response factor (SRF), and nuclear factor of activated T cells (NFAT); Exogenous Tax causes a significant shift of cells from G_0/G_1 to S and G_2/M phases of the cell cycle; directly inhibits the function or the expression of tumor suppressor genes. DNA polymerase *β* is downregulated by Tax, affecting both base excision repair and nucleotide excision repair. The telomerase reverse transcriptase (TERT) gene is activated by Tax, which also contributes to permanent cell proliferation and eventually transformation. Tax also induces defective mitotic spindle assembly checkpoint function, targets the RanBP1 protein, and causes abnormal amplification of cellular centrosomes, which causes chromosomal missegregation.
pX	Rex (p27)	Regulator of unspliced and singly spliced viral mRNA transport from the nucleus to the cytoplasm
pX	$p12^{I}$	Downregulation of major histocompatibility complex class I trafficking to the surface to evade CTLs; elevation of cytoplasmic calcium, increases T-cell activation. Increases NFAT activation, enhances LFA-1 mediated T-cell adhesion. Activation of STAT5b.
pX	p8	Cleavage product of p12; increases formation of intercellular conduits and viral transmission.
pX	$p30^{II}$	Interferes with TLR-4 signaling. Alters cell cycle and DNA repair. Promotes viral latency by interfering with *tax/rex* RNA export; stabilizes p53 and abrogates p53 suppressor cell function
pX	$p13^{II}$	Induces apoptosis; binds to and interferes with Tax transactivation; activates resting cells

ATL, adult T-cell lymphoma; CTL, cytotoxic T-lymphocyte; TLR, toll-like receptor.

strand of the pX region codes for the HTLV-1 bZip protein (HBZ) that downregulates viral transcription. Auxiliary proteins have been described in HTLV-2- and HTLV-3-infected cells (12, 13). HTLV-1 produces the HBZ (HTLV-1 basic leucine zipper [bZIP] factor) protein (14), while antisense transcription in HTLV-2 and the recently discovered HTLV-3 and HTLV-4 governs the production of a protein with some similarity in structure and function to HBZ, the Antisense Protein of HTLVs (APH), denoted APH-2, APH-3, and APH-4 for each virus, respectively (15–18). The HBZ protein is capable of inhibiting Tax-mediated activation of the HTLV-1 LTR, activating cellular transcription, and promoting T-lymphocyte proliferation (19, 20). The APH-2 and the APH-4 proteins localize in the nucleus of transfected cells, as does HBZ, while APH-3 localizes in both the nucleus and the cytoplasm. All APH proteins discovered thus far demonstrate the ability to repress Tax-mediated viral transcription (17, 18). While APH-2 is associated with a higher proviral load (PVL), it does not promote cell proliferation nor cause lymphocytosis (15, 16).

Biology

The replication strategy of the HTLVs involves a replication cycle typical of all members of the *Retroviridae* family, whereby the RNA genome undergoes reverse transcription into a DNA provirus that integrates into the host genome. Subsequently, new virions are produced via this integrated DNA template under the control of viral regulatory genes (Tax, Rex) (21).

Receptors

HTLV-1 preferentially infects CD4 T-helper cells, whereas HTLV-2 has a preferential tropism for the CD8 cell type (12). Dendritic cells are also infected by HTLV-1. Additionally, HTLV-1 infects a wide range of cells *in vitro*, including endothelial cells, fibroblasts, and CD8 T cells (but at

a lower frequency based on studies of T cells from patients with HAM) (22). HTLV-1 is transmitted through a viral synapse or a viral biofilm (23) and enters target cells via interaction with molecules (Glut1, Neuropilin-1, and heparin sulfate proteoglycans [HSPGs]) that form the receptor complex (24, 25). HSPGs act as attachment factors, Neuropilin-1 as the binding receptor, and Glut1 as the fusion receptor.

HSPGs are also required for the efficient entry of HTLV-1 (26). Higher levels of HSPGs are found on activated primary CD4 T cells compared to CD8 T cells. Conversely, CD8 T cells, the primary target of HTLV-2, express GLUT1 at dramatically higher levels than CD4 T cells, thus explaining the preference of HTLV-2 for CD8 cells. HTLV-2 surface glycoprotein binding and viral entry are markedly higher for CD8 T cells, while HTLV-1 surface glycoprotein binding and viral entry are higher for CD4 T cells rich in HSPGs. The infection of CD4 cells by HTLV-2 can be increased if CD4 cells are made to overexpress GLUT1, whereas the HTLV-1 infection of CD8 cells can be increased by using transfection to increase CD8 cell HSPG expression (26). Studies employing chimeric HTLV-1 and HTLV-2 viruses in which envelope sequences are swapped demonstrate that cellular tropism is determined by type-specific envelope mapping to the C-terminal portion of gp21, indicating that *env* is a major viral determinant for HTLV T-cell tropism (12). Neuropilin-1, the receptor for semaphorin-3A and VEGF-A165 and a member of the immune synapse, is a physical and functional partner of HTLV-1 Env proteins in HTLV-1 cell entry (27). Thus, both cellular and viral envelope factors account for the complex tropism of the HTLVs. Interestingly, the HTLV-3 receptor complex is distinct from but overlaps with those of HTLV-1 and HTLV-2 (28).

HTLV is preferentially transmitted via direct contact between infected and targeted cells through a structure referred to as the virologic synapse, which is formed between the envelope glycoprotein of the virus and a cellular binding partner (29) or through the transmission of a viral biofilm (23). It is hypothesized that early during infection, most new HTLV-1-infected cells are produced via this cell-to-cell synapse, resulting in a polyclonal infection of both CD4 and CD8 T cells. This model is consistent with epidemiological data suggesting that cell-free biological fluids rarely transmit HTLV-1. In later stages of infection, when an equilibrium between viral replication and the immune response is established, HTLV-1 mainly multiplies through mitosis of the host cells (30).

Replication

Once viral attachment takes place, the fusion of the virion with the cell membrane results in the uncoating of the diploid RNA genome of the virus. The virally encoded RNA-dependent DNA polymerase (reverse transcriptase) complexed to the genomic RNA of the virus transcribes viral RNA into double-stranded DNA. This double-stranded viral DNA is transported to the nucleus as a ribonucleoprotein complex where, through a complex process mediated by the viral integrase, insertion into the host genome occurs. The genomic integration of HTLV-1 establishes a lifelong infection and is integral to both the virus replication cycle and amplification through the expansion of HTLV-infected host cells (30).

Elements in the viral LTR are essential to integration and replication; they form the sites for covalent attachment of the provirus to cellular DNA and provide important regulatory components for transcription. Additional key regulatory elements of HTLV are *tax*, which activates transcription of the viral genome, and *rex*, which modulates the processing of the viral RNA expressing unspliced forms of the viral mRNA. The HBZ leucine zipper protein encoded by the antisense pX gene inhibits CREB-2 binding to the viral promoter, thus modulating the viral replicating impact of Tax (19). When the DNA provirus is expressed (transcribed by a cellular RNAII polymerase complex), viral genomic RNA, mRNA, and subsequently viral proteins are made by the cell. Under the influence of Rex, new genomic full-length viral RNA is assembled and packaged for release (budding). During the budding process, the envelope incorporates some of the cell's lipid bilayer, producing an infectious virion of about 100 nanometers.

EPIDEMIOLOGY

Molecular Epidemiology

The four recognized HTLVs are closely related to simian counterparts and are approximately 60% homologous with each other (Fig. 1) (4). Based on the proviral DNA sequence, HTLV-1 is composed of seven subtypes—termed A through G, with up to 10% sequence variation (31, 32). It is postulated that most of these lineages arose from separate interspecies transmissions from simians to humans as long ago as 10,000 years (33, 34). All subtypes except A have examples of simian T-lymphotropic virus (STLV) isolates from the same geographic area of predominance, supporting that HTLV-1 variation is derived more from separate simian-to-human historical transmissions rather than the evolution of HTLV-1s within humans (35). The Cosmopolitan (A) subtype has the widest distribution worldwide and consists of four subgroups. Subgroup a (Transcontinental) is present in Japan, the Caribbean, Colombia, Chile, and India; subgroup b (Japanese) is found in Japan and India; subgroup c (West African) is found in the Caribbean and Africa, brought to the Caribbean through the slave trade (36); and subgroup d in North African samples was recently shown to be a recombinant virus (37).

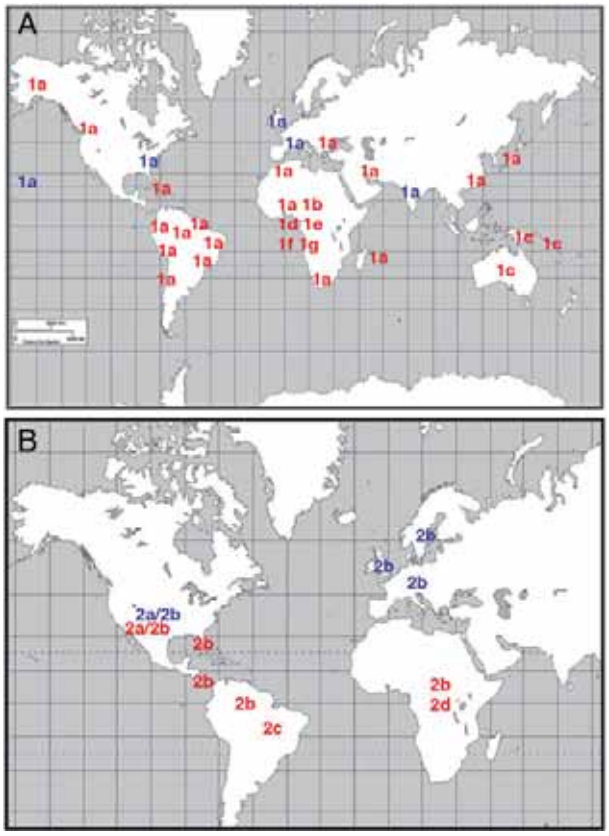
HTLV-2 consists of four types—A, B, C, and D—that are geographically distributed throughout the Americas and West Africa. Their prevalence is low in general populations but may be as high as 20% in Amerindian populations and drug user cohorts (38). Both HTLV-2 types A and B are found in West Africa and Cameroon. Between these subtypes, sequence differences of 2% to 4% are observed (39). In contrast to HTLV-1, HTLV-2 in humans appears to originate from simian-to-human transmission from a single simian species: bonobo apes (2).

More recently, HTLV-3 and HTLV-4 have been discovered in Central African pygmies (3,40–42). The full-length sequence of HTLV-3 suggests that it shares structural features that could make a pathogenic virus similar to HTLV-1, but no disease association has been made due to the limited number of infected humans (3, 4, 13,43–45).

Distribution

HTLV-1

HTLV-1 is widely disseminated (Fig. 3), and 5 million to 10 million people are estimated worldwide to harbor the HTLV-1 infection (32). Over the lifetime of the infected carriers, 2% to 4% will develop the aggressive T-cell malignancy ATL, and another 1% to 2% will experience chronic inflammatory diseases, mainly HAM (46). In some areas of endemicity, over 70% of all lymphoid malignancies are attributable to HTLV-1 exposure (47).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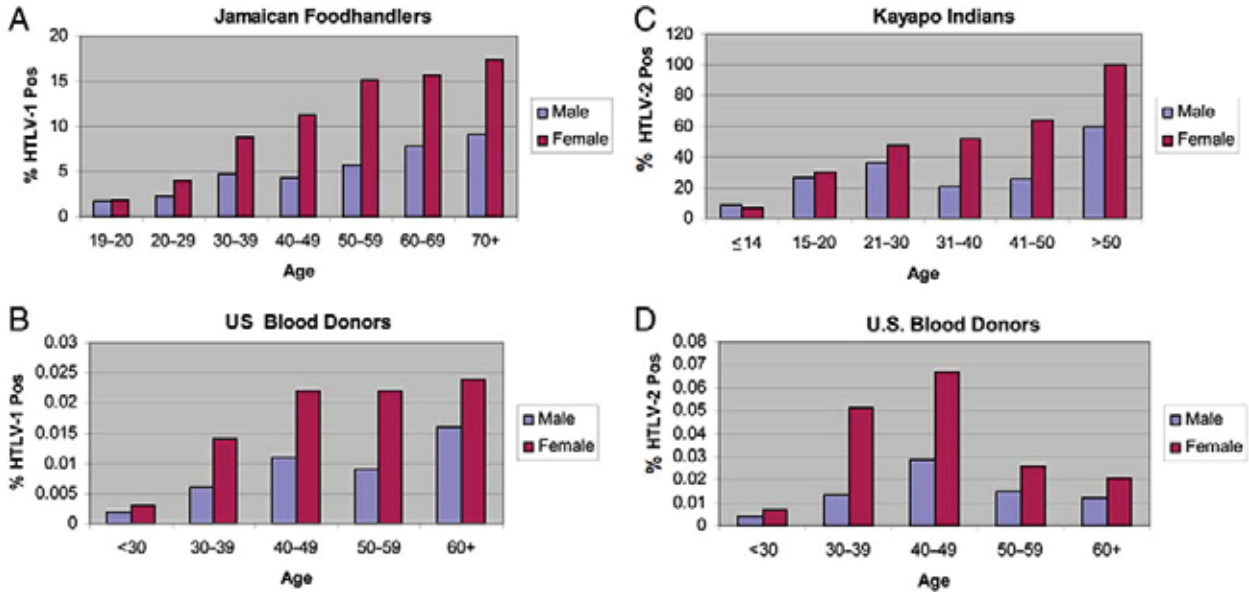

FIGURE 4 Seroprevalence of HTLV-1 (panels A and B) and HTLV-2 (panels C and D), illustrating age and sex dependence of HTLV-1 among endemic Jamaicans and US blood donors. For HTLV-2, a similar age and sex dependence is seen for endemic Kayapo Indians, but a likely birth-cohort effect is seen for US blood donors. (Adapted from references 51, 72, 214.)

risk factor for HTLV-2 in seropositive blood donors is previous sexual contact with an IDU (68).

Incidence and Prevalence of Infection

HTLV-1

HTLV-1 seroprevalence is characterized by an age-dependent increase that is observed in diverse geographic areas. The patterns of infection in Jamaica and in U.S. blood donors are similar despite orders of magnitude differences in seroprevalence (Fig. 4). The prevalence rate among children of both sexes is low (approximately 1%) and equal. During adolescence, coincident with the onset of sexual activity, the prevalence begins to rise, and a divergence in infection rates between males and females emerges, with female rates exceeding those of males. The reason for this female excess has been attributed to more efficient transmission from males to females. Part of this age-dependent rise, at least in Japan, has been attributed to a birth cohort effect whereby declining rates of infection in younger birth cohorts contribute to the observed age-dependent increase in prevalence (69). This pattern is observed in follow-up studies of Japanese emigrants to Hawaii, for whom rates of infection decline in successive generations (60). Possible explanations for this declining intergenerational prevalence include widespread condom use for contraception, changes in standards of living, improved nutrition, changes in breast-feeding patterns, the elimination of many coincident infectious diseases, and declines in other STDs. Nevertheless, Japanese and American couples discordant for HTLV-1 show evidence of ongoing person-to-person transmission (70, 71). There is no evidence to support the hypothesis that reactivation of immunosilent infection in seronegative persons accounts for this age-dependent increase in prevalence.

HTLV-2

HTLV-2 also exhibits a characteristic age-dependent rise in seropositivity in populations that are endemic (Fig. 4). Among populations of Amerindians in areas of endemic infection, prevalence increases with age similarly to HTLV-1; gender differences are less marked (72). Among IDUs, the sharing of injection equipment—a common practice before the wide availability of disposable syringes—explains the high rates of seropositivity in middle-aged and older persons compared to younger ones, again due to a birth cohort effect explained by high rates of drug use in the 1960s and the 1970s and fewer IDUs in subsequent decades. Again, there is little evidence of a virus-positive, antibody-negative state, although the HTLV-2 PVL is lower than that of HTLV-1.

Transmission

Table 2 summarizes the routes, modes, and cofactors associated with HTLV-1 and HTLV-2 transmission. There are fewer studies of the HTLV-2 transmission risk than of the HTLV-1 risk. Data on HTLV-3 and HTLV-4 are not available due to the small number of isolates thus far.

Routes

Mother-to-Child Transmission

The mother-to-child transmission of HTLV-1 is the principal means of childhood infection and is associated with the greatest risk of ATL. The risk of infection in children of seropositive mothers correlates with the provirus load in breast milk, the concordance of human leukocyte antigen (HLA) class I type between mother and child, and the duration of breast-feeding (73, 74). Among children breast-fed for a prolonged period, the rate of infection ranged between 15% and 25% (75).

In Japanese prevention trials, breast-feeding accounts for most occurrences of perinatal transmission. Although 20% of breast-fed infants seroconvert to HTLV-1, only 1% to 2% of formula-fed infants of HTLV-1-positive mothers become infected (76). Children usually seroconvert in the first 2 years of life; the rates remain stable until the early teenage years, when new infections occur via sexual transmission. Maternal antibodies, present during the first 6 months of the

TABLE 2 Transmission of HTLV-1 and HTLV-2

Mode of transmission or cofactor	HTLV-1	HTLV-2
Mode of transmission		
Mother to infant		
Transplacental	Low efficiency	Probable but not quantified
Breast milk	Very efficient	Probable but not quantified
Sexual		
Male to female	Very efficient	Yes but not quantified
Female to male	Efficient	Yes but not quantified
Male to male	Efficient	Not known
Parenteral		
Blood transfusion	Very efficient	Very efficient
Injection drug use	Efficient	Efficient
Cofactors		
Elevated virus load		
Mother to infant	Increased	Not known
Heterosexual	Increased	Increased
Sexually transmitted diseases	Increased	Not known
Cellular versus plasma transfusion products	Increased	Increased
Sharing of needles and paraphernalia	Increased	Increased
Cold storage of blood	Decreased	Decreased

child's life, appear to provide some protection for children exposed through breast milk, and infection rates increase as maternal antibody levels decline (77).

The viral load of the mother, as measured by antibody titer and viral antigen level in breast milk or blood, is the best predictor of the risk of mother-to-infant transmission (77), with the risk of transmission found to increase from 4.7 per 1,000 person-months when the provirus load in breast milk was less than 0.18% to 28.7 per 1,000 person-months when it was greater than 1.5% (74). The presence of antibodies to the Tax antigen and antibodies to certain envelope epitopes is associated with enhanced transmission. These antibodies are a surrogate for elevated maternal viral load, the major risk factor for transmission (77).

Limited studies of the mother-to-child transmission of HTLV-2 infection have been performed to date. The detection of HTLV-2 in breast milk, the 1% to 2% prevalence rate among preadolescent Guaymi Indians (a noninjecting drug use cohort from Panama at high risk for HTLV-2), and the finding that seropositive children had seropositive mothers support this route of infection (78).

Sexual Transmission

HTLV-1 is present in the genital secretions of infected individuals and can be transmitted through sexual intercourse. A Japanese cohort of discordant couples followed for 10 years found that 27.3% of the initially seronegative women seroconverted, compared to 6.7% of the initially seronegative men, indicating that the risk of male-to-female transmission was 4.2 times higher (71). A similar study among former US blood donors found higher rates but similar gender patterns of HTLV-1 and HTLV-2 transmission from men to women (1.2 transmissions per 100 person-years) as from women to men (0.4 transmissions per 100 person-years) (71).

Markers of sexual activity, such as an increased number of sexual partners and contact with female prostitutes, are associated with HTLV-1 transmission, as are STDs, such as syphilis and genital ulcer disease. The duration of a steady sexual relationship with a seropositive partner is also associated with seroconversion. Elevated HTLV-1 load, as measured by antibody titer or quantitative PCR of blood samples, correlates with a heightened risk of sexual transmission (79). The presence of anti-Tax antibody (perhaps a measure of *in vitro* proliferation) has also been associated with transmission (80).

The sexual transmission of HTLV-2 has been difficult to study because of the frequency of coincidental injection drug abuse among study populations (e.g., drug-addicted female prostitutes) (81). However, one prospective study of discordant couples found rates of HTLV-2 transmission (0.5 transmissions per 100 person-years) similar to those of HTLV-1 (0.9 transmissions per 100 person-years) (71). Among Amerindian populations the sexual transmission of HTLV-2 was documented in cross-sectional studies showing high concordance rates for seropositivity in couples (82).

Parenteral Transmission

Parenteral transmission by transfusion, transplantation, or drug injection is an efficient means of acquiring HTLV infection. Although the probability of acquiring the infection through transfusion has been significantly reduced by blood donor selection and HTLV antibody screening, the transfusion of contaminated cellular components can result in seroconversion in more than 40% of recipients (53). The filter-based leukoreduction of blood products may reduce this risk substantially but not to zero (83). Among US blood donors who are confirmed to be HTLV positive, approximately half are HTLV-1 positive, and half are HTLV-2 positive, with regional differences. Their major risk factors for infection are sexual contact with an IDU, injection drug use, and birthplace in a region where HTLV-1 is endemic. The use of immunosuppressive drugs, such as corticosteroids, by the recipients of blood components may heighten susceptibility by blunting the cellular immune response to HTLV-1 following exposure. The transmission of HTLV-2 has been well documented for 50% of the recipients of known units of positive blood (84).

HTLV has been considered a contraindication to organ donation, but according to the United Network for Organ Sharing database, the prevalence of HTLV-1 or HTLV-2 infections in organ donors is 0.027% and 0.064%, respectively. In 2007 no reports of HTLV-related diseases in recipients were reported in a cohort at 1 year posttransplantation (85). The screening of deceased donors was discontinued in the United States in 2009. However, a recent report of HTLV-1 transmission and HAM following renal transplantation questions the wisdom of this decision (86). HTLV-1 transmission by organ transplantation has also been reported in Germany (87).

Parenteral drug abuse has been associated with the transmission of both HTLV-1 and HTLV-2, but most HTLV-positive drug abusers are infected with HTLV-2. This is likely due to epidemiological factors, namely historical transmission from Amerindians endemic for Native American HTLV-2 source and amplification by IDUs (88). Risk factors for seroconversion include the sharing of drug paraphernalia and blood exposure, which was more common before the wide availability of disposable syringes. Needle exchange

programs instituted to stem the HIV epidemic have likely contributed to a decrease in HTLV transmission among IDUs, although this has not been proven.

Environmental and Socioeconomic Factors

There is no evidence of a seasonal variation in HTLV infection. Given the relative inefficiency of HTLV-1 transmission and its association with lymphoid cells, vector transmission is not thought to occur (51). The association of HTLV-1 with markers of lower socioeconomic status, such as poor housing, hygiene, and nutrition, are more likely due to behavior than environmental factors.

Occupational infection with HTLV appears to be rare. Documented cases include a health care worker who seroconverted to HTLV-1 after experiencing a "microtransfusion" when a syringe loaded with blood punctured his foot (89) and the transmission of HTLV-2 to a laboratory worker from a needlestick injury as she recapped a syringe after collecting material for an arterial blood gas analysis (90). In another study, an HTLV-2-infected blood donor admitted no risk factors except his work as a dentist (91). Casual contact does not seem to be sufficient for transmission.

PATHOGENESIS

HTLV-1 is associated with two major categories of disease: (i) an aggressive CD4 T-cell leukemia/lymphoma called ATL and (ii) a chronic inflammatory neurologic disease, HAM, which is associated with inflammation and fibrosis of the long motor tracts of the spinal cord. In the case of ATL, HTLV-1 is monoclonally integrated into the leukemic cells, whereas in HAM, neurologic damage appears to result from an overexpression of virus and an altered immune response. Current research has demonstrated no specific genomic integration site associated with ATL (92). At the center of disease pathogenesis are the gene products of the pX reading frame (*p*X I to *p*X IV in Fig. 2 and Table 1), which not only engage in promoting viral replication but also impact cellular functions that favor viral replication. The most studied of these viral gene products coded for by pX IV is the p40 Tax viral regulatory protein, which, like its counterpart Tat from HIV-1, plays an important role in promoting viral growth and disease pathogenesis (93–95). More recently, HBZ has been recognized for its crucial role in the proliferation of ATL cells and the induction of T-cell lymphoma and systemic inflammation, mainly through the regulation of LTR transcription and the modulation of cellular signaling associated with cell growth, T-cell differentiation, and the immune response. The additional accessory proteins coded for by the pX region also play a role in pathogenesis (11).

Viral Factors

Effects of Tax Protein

Tax is a posttranslationally modified protein (93) that shuttles from cytoplasm to nucleus and is responsible for the activation of virus transcription via *tax*-responsive elements. Tax alters cellular gene transcription and activates the proto-oncogenes c-*fos* and c-*erg*, as well as an array of early response genes, the human lymphotoxin gene, and the parathyroid hormone-related protein gene while it transrepresses the *β*-polymerase gene (96, 97). Tax alters the type I interferon (IFN-I) pathway (98), and the overproduction of interferon-gamma has been implicated in promoting the chronic inflammation that characterizes diseases such as HAM. Tax also affects regulators of the cell cycle, leading to uncontrolled cell proliferation that promotes the potential for oncogenic transformation and unregulated immune control and stimulates cell growth by directly inhibiting the function or the expression of tumor suppressor genes (99). Tax functions as an oncoprotein both to induce proliferation by the mechanisms summarized above and to inhibit apoptosis by activating the expression of BclXL and of Bfl1 (100, 101), as well as by direct transcriptional transactivation of cellular regulators of apoptosis (102, 103). Another strategy involves Tax making HTLV-infected cells resistant to various physical, chemical, and biological inducers of apoptosis, which may contribute to the risk of malignant transformation because such cells survive with damaged DNA following environmental carcinogenic exposures (104). Tax also induces structural DNA damage and alters chromosomal stability, resulting in harm to the cell's genomic integrity on the path to malignant transformation (Fig. 5). The high frequency of aneuploidy observed in ATL appears to result from Tax directly binding to mitotic arrest deficiency protein 1 (MAD1) and disturbing the segregation of chromosomes during mitosis (105). Given the high frequency of aneuploidy in ATL, the transition from oligoclonal expansion to malignant transformation is brought about by the impact of Tax on chromosomal stability (99, 106).

Effects of HBZ Protein

HBZ was initially identified as a binding protein that interacted with CREB/CREB-2 and inhibited HTLV-1 transcription (14, 107). HBZ promotes T-cell proliferation in its mRNA form, whereas it suppresses Tax-mediated viral transcription through the 5′ LTR while in protein form (108). The HBZ binding protein, ATF3, is highly expressed in ATL cells, and its negative modulatory effects are curtailed by HBZ, allowing the proliferation of ATL cells via upregulation of CDC2 and cyclin E2 (109). HBZ enhances the transcription of telomerase reverse transcriptase, which in turn has been associated with the formation of malignant tumors, thus HBZ is implicated in ATL oncogenesis (110), and HBZ transgenic mice develop lymphoma (111). HBZ suppresses the NF-*k*B pathway and impairs cell-mediated immunity (112). It has been postulated that HTLV-1 might escape host immune attack through this mechanism (19). HBZ also allows HTLV-1 to convert infected T cells into Tregs by enhancing transforming growth factor β (TGF-β) expression. This is believed to be critical for virus persistence (19). Thus far, HBZ has been shown to inhibit Tax-mediated activation of the HTLV-1 LTR, activate cellular transcription, and promote T-lymphocyte proliferation, while all APH proteins have demonstrated the ability to repress Tax-mediated viral transcription (14, 15, 17, 18, 113). In HTLV-2, although APH-2 is associated with a higher PVL, it has not been shown to promote cell proliferation or lymphocytosis (15, 16).

Effects of pX I and II Proteins

pX I produces $p12^{I}$, which is essential for establishing persistent infection (11). $p12^{I}$ targets cellular pathways involved in T-cell proliferation, thus contributing, along with Tax, to T-cell activation. $p12^{I}$ also interferes with the trafficking of the heavy chain of major histocompatibility complex (MHC) class I to the surface of T cells, thus contributing to the persistence of infected cells by blocking immune recognition. p12 can be cleaved into p8, which is involved in viral transmission (10). Efficient viral persistence and transmission requires both p12 and p8 (114). pX II produces $p13^{II}$ and $p30^{II}$. p13 activates resting cells and

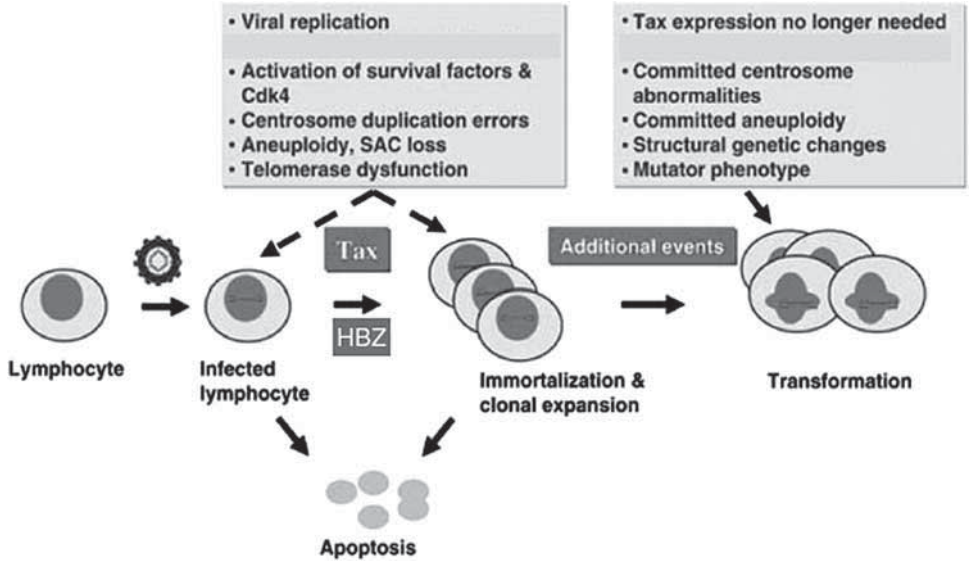

FIGURE 5 HTLV lymphomagenesis: progression of T lymphocytes from immortalization and clonal expansion to transformation. Various changes in the cell during the different stages are listed in the shaded boxes. As shown, virus-infected cells can also go into apoptosis. (Adapted from reference (99) with permission of Macmillan Publishers Ltd.)

promotes apoptosis in transformed cells (115). $p30^{II}$ alters cell cycle and DNA repair mechanisms and promotes viral latency. Consequently, it decreases Tax expression and Tax-mediated viral transcription. The interaction between $p30^{II}$ and Rex appears to govern the switch between virus latency and replication (116).

Proviral Load

Until molecular amplification tools became available, it was assumed that HTLV-1 had a low rate of expression because levels of plasma-associated virus were undetectable, and the variation in sequence diversity around the world was minimal. However, the cell-associated PVL of HTLV-1 is remarkably high compared to that of other retroviruses (117). Quantitative PCR shows that the median PVL among healthy carriers involves between 0.1% and 1.0% of peripheral blood mononuclear cells (PBMCs) (118). The number of infected cells present in the blood of a healthy carrier is generally relatively low; mean PVLs are 3.28 $\log^{10}$ copies per million PBMCs (range, 0.5 to 5.3) for HTLV-1 and 2.60 $\log^{10}$ copies per million PBMCs (range, 0.05 to 5.95) for HTLV-2 (119). In patients with HAM, between 5% and 10% of peripheral blood cells are infected, and in rare cases 30% of peripheral blood cells harbor the virus. Careful molecular analysis of HTLV-1 in various disease states has indicated that HTLV-1 infection is largely an oligoclonal rather than a polyclonal expansion. These observations are consistent with the hypothesis that most HTLV-1 replication and expansion occur via the proliferation of infected cells containing integrated provirus rather than through infection by cell-free virions. As a consequence, discussed below, cell-mediated immune responses are key for controlling viral infection (120). HTLV-2 genome-wide integration sites are similar to HTLV-1, but the expansion of HTLV-2-infected clones does not appear to have the same association with the genomic environment of the integrated provirus. Recent work has demonstrated that PVL in HTLV-2 is almost exclusively confined to $CD8^+$ T cells and is composed of a small number of highly expanded clones, which suggests there are significant differences in the selection forces that control the clonal expansion of HTLV-1 and HTLV-2 during infection (120).

Host Immune Responses

In the face of prodigious mechanisms for the expansion of viral infection, a robust and targeted cell-mediated immune response is mounted to control HTLV infection. In most cases this response is effective, given the relatively infrequent occurrence of disease among carriers, but at times the immune response may contribute to disease occurrence. A proposed model of this viral interaction with the host cell-mediated immune response is shown in Fig. 6.

Innate Immunity

The main *in vivo* cellular targets of HTLV-1 are cells from the adaptive immune system, for example, CD4 and C8 T cells. However, innate immune cells (monocytes, macrophages, and DCs) are permissive to the virus *in vitro* and/or are infected *in vivo* (94). The role of the innate immunity in HTLV-1 pathogenesis is not clear; however, HTLV-1-infected cells have been shown to alter IFN-I signaling through the Tax protein, and the addition of IFN-I efficiently suppresses HTLV-1 replication *in vitro* (98). However, even if it does not promote cell cycle arrest or cell death *in vitro*, the alpha interferon (IFN-α)-azidothymidine (AZT) combination is particularly efficient for treating leukemic, smoldering, and chronic ATL patients and significantly improves their survival.

Humoral Immune Responses

Antibodies to the various antigens of HTLV-1 occur at high levels in carriers and among patients with ATL and HAM. During primary HTLV infection, the first specific antibodies to emerge are directed against the Gag proteins. Subsequently, anti-Env antibodies appear, and about 50% of infected individuals develop detectable antibodies to p40 Tax protein (80). An older study found a median time to seroconversion of 51 days following transfusion-transmitted

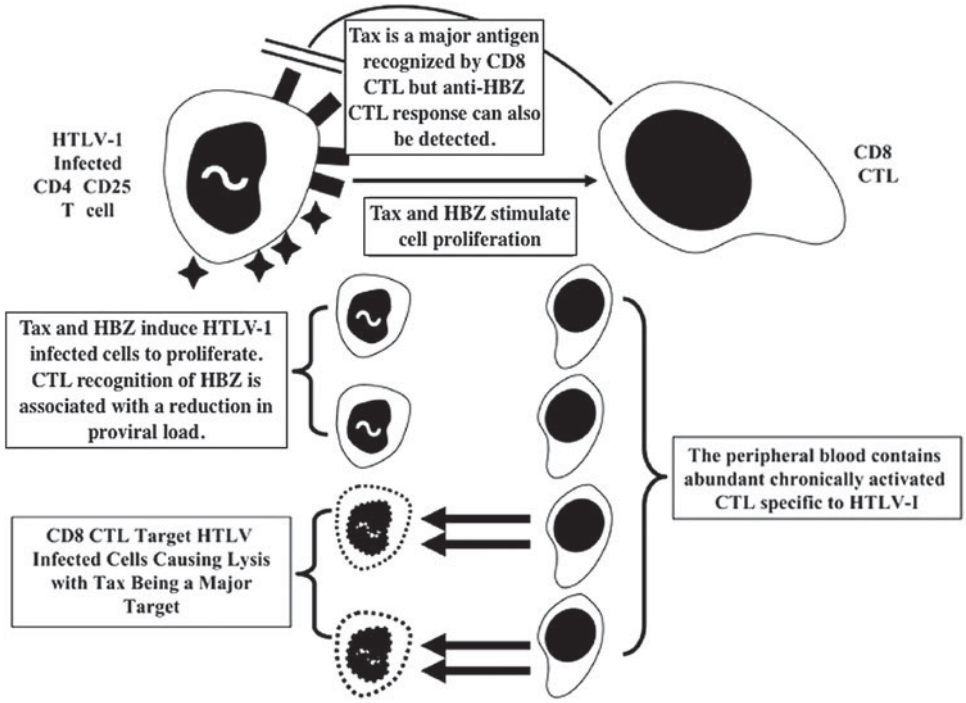

FIGURE 6 Model of CD8 CTL-mediated control of HTLV-1 infection. HTLV-1 infects CD4 T lymphocytes, with expansion of infection primarily via cell replication. As HTLV-1-specific antigens, particularly Tax, are expressed, a robust CD8 CTL response is generated that results in the killing of CD4-infected cells expressing Tax. Anti-HBZ CTL response is weak but associated with a reduction of PVL. The inability of some persons to control HTLV-1 expansion is thought to contribute to disease pathogenesis. In this model, the dynamic equilibrium between viral replication and immune destruction is mediated through Tax overexpression, causing CD4 target cell proliferation and a robust cell-mediated response to the antigen in particular, thereby leading to CTL-mediated lysis of these HTLV-1-infected CD4 cells. (Adapted from references (121, 123, 215, 216.) As a consequence of ongoing Tax and/or HBZ expression, there is a cell-associated expansion of HTLV-1 genome-containing CD4 cells and a compensatory expansion of CD8 CTL. As the number of CD4 cells containing integrated HTLV-1 provirus expands, HTLV-1 antigens are expressed on the cell surface and become targets for CD8-mediated cytotoxic killing.

infection, but time to seroconversion is likely to be shorter with newer, more sensitive antibody assays (53). Antibody titers vary from patient to patient, are significantly higher in patients with HAM and among those at risk for this disease, and correlate with the proviral burden (77, 118). This may explain the paradoxical finding of high antibody titers among women who transmit HTLV-1 to their infants through prolonged breast-feeding (75). The explanation for this paradox is that transplacental maternal antibodies appear to protect the infant from infection in the first months of life, but as passively acquired antibodies wane the infant becomes infected via maternal virus transmitted via breast milk, most likely by cell-associated infection. Other than this apparent protection afforded the baby through passive antibody transfer from an infected mother at birth, few data suggest that humoral immune responses play a role in protection from disease. The high titers observed in patients with HAM and ATL seem to reflect immune responses in the context of high viral burden observed in these conditions rather than as a direct correlate of disease risk.

Cell-Mediated Immunity

Cytotoxic T lymphocytes (CTL) targeted at viral antigens expressed on infected cells play an essential role in the regulation of viral expression. Among chronic carriers, infected individuals mount a strong cell-mediated immune response to the virus, and up to 1% of $CD8^+$ CTL can recognize at least one epitope of HTLV. Freshly isolated cells have substantial expression of activation markers, indicating that these cells have recently encountered the Tax antigen (121–123). Cell-mediated immune response to HTLV may also play a role in the pathogenesis of HAM.

In the case of HAM, there is an imbalance in immune response manifested by an overexpression of Tax, high levels of Tax-specific CTL, and the excessive elevation of HTLV viral load. For instance, CXCL10/IP-10, a T-helper type 1 (Th1)-associated chemokine, has been found to be significantly elevated in the cerebrospinal fluid (CSF) of HAM patients compared to healthy controls (124). Other cytokines in the Th1 pathway such as transcription factors T-beta, GATA-3, IL-12Rß2, and suppressors of cytokine signaling are also markedly elevated in HAM patients (125). The role for CD8-mediated cell killing as the primary means of viral suppression may explain the epidemiological observation that recipients of infected blood products who are also receiving exogenous immunosuppressive medications are more susceptible to HTLV-1 infection due to the host's inability to clear the initial virus infection (54). CD4 CD25 T-cell subsets, the target of HTLV-1 infection (Fig. 6), stimulate and expand HTLV-I Tax-specific CD8 T cells, which may play an important role in the pathogenesis of HTLV-1-associated neurologic disease (126). The finding that Tax1A is more frequent in HAM than Tax1B may point to a viral motif in Tax1A that contributes to pathogenesis. Such a mechanism is consistent with the hypothesis that there is molecular mimicry between a viral and a cellular antigen. This

hypothesis is supported by the finding that HAM patients make antibodies to heterogeneous nuclear ribonuclear protein A1 (hnRNP A1), a neuron-specific autoantigen (127). Monoclonal antibodies to Tax cross-react with hnRNP A1, showing molecular mimicry between the two proteins and providing a pathogenic basis for autoimmune neurologic disease. Additionally, the finding of oligoclonal immunoglobulin bands in the CSF of HAM patients who react to HTLV-1 antigens supports this hypothesis (128).

Impact of CD8 Lymphotropism of HTLV-2

In contrast to the predominant CD4 lymphotropism of HTLV-1, HTLV-2 provirus *in vivo* is integrated at the highest levels into CD8 lymphocytes but may also be demonstrated in CD4 cells (12, 129). The delayed hypersensitivity response is normal among HTLV-2-infected individuals, suggesting intact cell-mediated or Th1-type immunity (130). HTLV-2 infection is associated with chronic elevations in absolute lymphocyte and platelet counts (131). As noted above, there are differences in the clonal distribution of HTLV-1 and HTLV-2, and the PVL in HTLV-2 is almost exclusively confined to $CD8^+$ T cells and is composed of a small number of highly expanded clones (120). Total immunoglobulin G levels are higher in HTLV-2-infected persons (132), and HTLV-2 may induce the expression of gamma interferon, granulocyte macrophage colony-stimulating factor, and other cytokines (133, 134). Although HTLV-2 provirus has also been demonstrated in macrophages (135), whether such infection influences macrophage regulation or function to a clinically notable degree is not known. More intensive diagnostic evaluation and follow-up of HTLV-2-infected individuals is needed to confirm the relationship between HTLV-2 and recurrent pneumonia or asthma and other diseases (136).

Pathogenesis of ATL

Following initial infection, there is a proliferation of infected cells that is downmodulated by CTL immune responses. Oligoclonal infection of mainly CD4 postthymic T cells manifests within months, and the infected cells resemble transformed but not malignant cells (79). In some instances, monoclonal infection of T cells is observed. Such oligoclonal and monoclonal expansions have been noted to appear and then sometimes disappear spontaneously.

The effect of Tax on ATL pathogenesis appears to be mediated via the transactivation of a variety of cellular growth (transcription) factors and bioactive cytokines, the interruption of cell cycle regulatory factors resulting in interruption of cell cycle arrest, the blocking of normal apoptotic pathways, and the interference with DNA repair (Fig. 5) (95). However, Tax expression is not necessary for maintenance of the malignant genotype because in most ATL cases, Tax is not expressed (108). Rather, evidence supports the HBZ protein encoded by the minus strand of HTLV provirus as being critical in the development of ATL (17). The *HBZ* gene remains intact and is consistently expressed in all ATL cases (19). HBZ promotes the proliferation of ATL cells and induces T-cell lymphoma and systemic inflammation, mainly through the regulation of LTR transcription and the modulation of cellular signaling associated with cell growth, T-cell differentiation, and the immune response. Interestingly, HBZ could be a target for immunotherapy (137, 138).

Thus, for ATL, the net effect of Tax, HBZ, and other ORF products is that the normal checks to transition between cell cycle phases are abrogated (Fig. 5), contributing to the potential for heightened mutation. Genetic damage that would normally be repaired accumulates, apoptotic cell death does not occur, and Tax induces aneuploidy directly—all in the path to malignancy. Subsequently, T cells can accumulate DNA mutations, resulting in transformation and the monoclonal outgrowth of a truly malignant cell. At the stage of malignancy, the *tax* gene is silent, and Tax is no longer expressed, as transformation of the malignant cell is complete.

Pathogenesis of HAM

The pathogenesis of HAM appears to involve the dysregulation of immune function. This condition and other diseases with "autoimmune" manifestations of HTLV-1, coupled with the inability to downregulate HTLV-1 expression, appear to result from a failure of the cell-mediated immune response in HAM among carriers at risk. In the case of HAM, the major pathological manifestations appear to result from collateral damage to neurologic tissues as part of a cell-mediated immune assault on abundant CD4 HTLV-1-containing cells that infiltrate the central nervous system (CNS) (139). Elevated levels of lymphokines such as IL-6, tumor necrosis factor beta (TNF-β), and IL-2 are found in the CSF. However, attempts to document the presence of HTLV-1 in demyelinated lesions have not demonstrated a direct role for the virus in the target cell.

It is possible that HTLV-1 induces an autoimmune-like process through molecular mimicry or by indirect effects on immune function (see above). Autoantibodies against nuclear and perinuclear human brain proteins cross-reacting with different HTLV-1 epitopes have been found in the sera of HAM patients (140). Virus loads are high in HAM, suggesting a deficiency in the ability of the host to control viral proliferation. Furthermore, the pattern of oligoclonal expansion observed in healthy carriers is amplified in HAM. Up to one in five peripheral blood cells is infected with a high proportion of $CD8^+$ CTL targeting HTLV-1. Immunogenetic factors are associated with disease and possibly the overexpression of the virus (141).

The finding of HTLV-1 in CD8 cells and the coincidence of CD8 cells in spinal cord lesions raise the possibility that these cells may contribute to the induction of local spinal cord damage. Alternatively, because CTL against HTLV-1 account for a significant percentage of such lymphocytes, neuropathology has been hypothesized to result from collateral damage due to the local overproduction of harmful cytokines secreted by these cells. Thus, the tissue damage in the CNS in patients with HAM may be caused by lymphocytes chronically activated by the HTLV-1 Tax protein and secreting harmful cytokines and metalloproteinases (142, 143). The CD4 CD25 T-cell population is the main reservoir for HTLV-1 in HAM patients (144).

An association between class I HLA haplotypes and protection against HAM suggests that carriers of certain antigen-presenting motifs augment the efficient control of HTLV-1-containing cells. Thus, carriers of the HLA-A*02 haplotype are less prone to developing HAM. Non-HLA-A*02-positive carriers may be at increased risk of HAM because of an impaired cell-mediated immune response against HTLV-infected cells, resulting in the overexpression of HTLV (145, 146). Additionally, HLA-DRB1*0101 doubles the odds of HAM in the absence of the protective effect of HLA-A*02 (146). These data suggest that deficient immune recognition of Tax epitopes blunts a robust cell-mediated immune response.

Pathogenesis of Other HTLV-Associated Conditions

For other HTLV-1-associated autoimmune diseases, such as uveitis and arthritis, HTLV-1-infected cells at the site of pathological changes and lymphokine and cytokine overexpression may account for the local pathological manifestations (147).

LABORATORY DIAGNOSIS

Virus Isolation

The isolation of HTLV by culture is used only in the context of research studies and is not a routine diagnostic procedure. The technique employs the cocultivation of patient cells with human PBMCs that have been stimulated *in vitro* with mitogens (e.g., phytohemagglutinin) and growth factors (e.g., IL-2). When peripheral blood from patients with ATL is cultured, the virus-positive cell that emerges has a normal karyotype, and the tumor cells themselves do not grow. The ability to isolate HTLV is dependent on the viral load, the immune status, and the stage of disease. Because HTLV-1 is so strongly cell associated, plasma antigenemia is not present at detectable levels. Antigen assays are used for detecting virus antigen in the supernatant of short-term cell cultures.

Cellular Assays

In the research setting, the detection of cellular immune responses to HTLV-1 involves standard CTL assays using chromium release by either leukemic cells or transformed cells treated with HTLV-1 peptides or infected with recombinant virus. Flow cytometry-based assays adapting tetramer technology provide a much more precise measure of cell-specific CTL quantities. In these assays, PBMCs can be directly quantitated by the ability of MHC class I-restricted tetramers to bind Tax peptides (121).

Nucleic Acid Detection

PCR assays previously employed in the research setting to confirm HTLV infection, distinguish HTLV type, and quantify the level of virus under various clinical conditions (148) are now available commercially to clinicians in practice. Refinements of the PCR assay resulted in a highly sensitive and reproducible detection system with limits of detection at 10 molecules of DNA in 1 milligram of human DNA (about 1 infected cell in 100,000 PBMCs) (117, 149). In the research setting, quantitative PVL measured by real-time PCR may be useful as a prognostic marker for disease progression in infected patients, and PCR following reverse transcription is used to detect viral mRNA in infected cells. Qualitative PCR detection of HTLV provirus is increasingly being applied in the clinical setting, for example, to distinguish a false-positive antibody test or to confirm infection in the context of transplantation. However, the tendency of PCR to yield false-positive results should dictate caution in its clinical use. For research applications, flow-based systems have allowed the detection of cell-specific viral antigen expression, usually Gag- or Tax-specific, in subsets of T lymphocytes (121).

Serologic Assays

The primary test for HTLV-1 or HTLV-2 infection is detection of virus-specific antibody by a variety of techniques. Because HTLV infection is chronic, almost all HTLV-1 antibody-positive patients are also positive for HTLV proviral DNA. The most widely used assay for detection of HTLV-1 antibodies in the United States is the enzyme immunoassay (EIA). In Japan a particle agglutination assay is used. These assays are highly sensitive and specific for virus infection (150), but the EIAs for HTLV-1 and HTLV-2 are cross-reactive. The virus subtype is distinguished by Western blot technology, which uses a combination of whole virus and recombinantly produced peptides. This assay is used to confirm that an EIA sample is truly positive and to distinguish between HTLV-1 and HTLV-2 types (151). The criterion for Western blot confirmation is the presence of antibody to the p24 *gag* gene product and one of the HTLV-1 *env* gene products, p21e (transmembrane), gp46 (external envelope), or gp6l (whole envelope). Sera with no reactivity to viral protein bands are considered negative; sera with partial reactivity are called indeterminate. Additional approaches such as the line immunoassay (LIA) or the recombinant immunoblot assay (RIBA) are supplanting the Western blot (Fig. 7). These assays contain both group-specific conserved antigen motifs from the transmembrane protein and type-specific antigen motifs from the external glycoproteins (recombinant gp46) of HTLV-1 and HTLV-2. The antigens are coated onto the strips and allow the simultaneous confirmation and differentiation of both HTLV-1 and HTLV-2 in 98% of cases (152). Confirmation by immunofluorescence assay (IFA) may be used but is labor-intensive, operator-dependent, and requires the maintenance of infected cell lines as reagents by reference laboratories. Titer determination to allow the quantification of antibody is possible with modifications to the EIA, the particle agglutination assay, and the IFA.

In tropical countries, particularly Africa, repeatedly reactive EIA samples exhibit a high frequency of indeterminate Western blot results. Typically, these sera show an HTLV Gag indeterminate profile, which may be related to endemic *Plasmodium falciparum* infection (61). To avoid overestimating the rate of HTLV-1 and HTLV-2 seroprevalence in these regions, PCR may be useful (124–126).

CLINICAL MANIFESTATIONS

Acute seroconversion is associated with no recognized clinical syndrome. The median time from infection to seroconversion has been estimated at 51 days in a study of transfusion transmission, although this serologic window is likely shorter with more sensitive contemporary antibody assays (53). The time from seroconversion to disease can vary from 18 weeks with HAM to many decades with ATL. As summarized in Table 3, a wide range of clinical conditions are linked to HTLV-1, some of which result from virally induced cell transformation, as in ATL, while others appear to result from the indirect effects of virus-induced immunologic perturbation.

ATL

Before the discovery and isolation of HTLV-1, ATL was characterized as an aggressive leukemia/lymphoma of mature T lymphocytes, with varied clinical manifestations: generalized lymphadenopathy, visceral involvement, hypercalcemia, cutaneous skin involvement, lytic bone lesions, and leukemia cells with pleotrophic features. Almost all patients with ATL present with lymphadenopathy, and 50% have hepatosplenomegaly (127).

The Lymphoma Study Group in Japan has classified ATL into four clinical types based on clinical features and cell morphology: acute, chronic, smoldering, and lymphoma/leukemia types (145). Figure 8 shows the characteristic

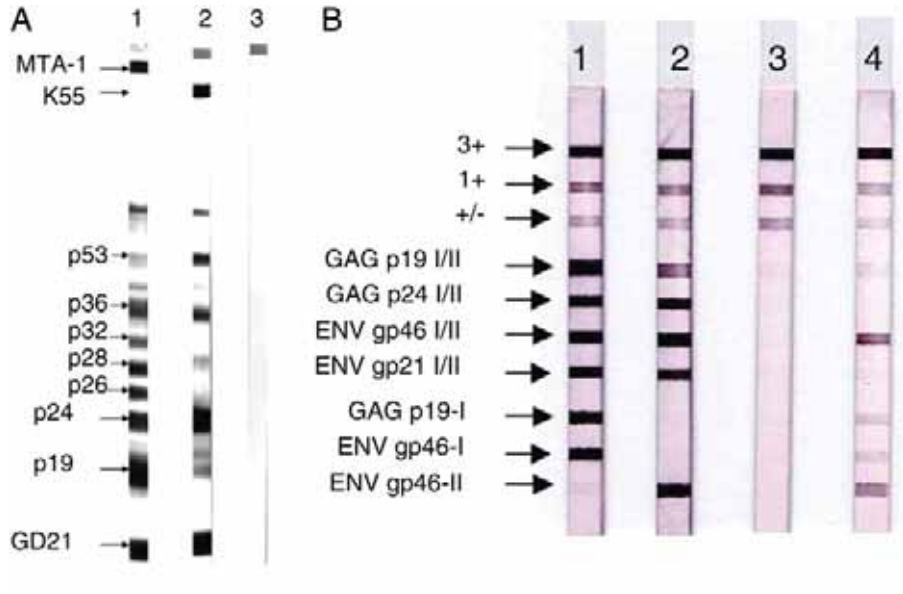

FIGURE 7 Examples of assay results for HTLV-1 and HTLV-2. (A) Modified HTLV-1 and HTLV-2 Western blot including recombinant type-specific peptides MTA-1 for HTLV-1 and K55 for HTLV-2, in addition to other viral antigens. Lane 1 shows an HTLV-1-positive serum, lane 2 shows an HTLV-2-positive serum, and lane 3 shows a negative control serum. Modified from reference (217). (B) HTLV-1 and HTLV-2 line immunoassay, including type-specific HTLV-1 GAG p19, HTLV-1 ENV gp46, and HTLV-2 ENV gp46 antigens, in addition to shared GAG p19, GAG p24, ENV gp46, and ENV gp21 antigens. Lane 1 shows an HTLV-1-positive serum, lane 2 shows an HTLV-2-positive serum, lane 3 shows a negative control serum, and lane 4 shows an HTLV-3-positive serum from an African pygmy. (Modified from reference 42.)

morphological features of the leukemia cells and examples of the cutaneous manifestations that may be the first indication of disease. The prototypic acute ATL is distinguished by increased numbers of leukemic T cells with characteristic pleomorphic morphology (Fig. 8A), skin lesions (Fig. 8D), systemic lymphadenopathy, hepatosplenomegaly, and metabolic disorders, especially hypercalcemia.

Chronic ATL (Fig. 8B) resembles chronic T-lymphocytic leukemia; cells have a characteristic cleaved morphology called buttock cells, the white blood cell count is increased, and skin lesions are evident. Some patients manifest mild lymphadenopathy and hepatosplenomegaly, and the serum lactate dehydrogenase level is sometimes elevated. Hypercalcemia and hyperbilirubinemia are not characteristic of this type of ATL.

Smoldering ATL (Fig. 8C) may clinically resemble mycosis fungoides/Sézary syndrome, with cutaneous involvement manifesting as erythema or as infiltrative plaques or tumors (Fig. 8D), and Pautrier's microabscesses characteristic of mycosis fungoides may be observed. This was the case in the first patient from whom the virus was isolated in the United States by Gallo and colleagues (146).

In patients with chronic or smoldering ATL, a long prodrome of symptoms or signs is usually noted before transformation to an acute ATL. Sometimes, ATL manifests as a non-Hodgkin's T-cell lymphoma with no other clinical features of ATL except monoclonal integration of HTLV-1 in the proviral DNA of the tumor cells. These cases are termed lymphoma-type ATL and are indistinguishable from peripheral T-cell lymphomas.

Between 50% and 60% of ATL patients have the acute type, 20% have the chronic and lymphoma type, and 5% have the smoldering type. Most patients with the acute and lymphoma types die within 6 months of diagnosis in the absence of therapy (Fig. 9), particularly if hypercalcemia is a presenting sign. In general, the smoldering type is the least aggressive; patients with the chronic type have a relatively poor prognosis, with death occurring within a few years of diagnosis. The cause of death is usually an explosive growth of tumor cells, hypercalcemia, bacterial sepsis, and other infections observed in patients with immunodeficiency.

Opportunistic infections are often present and contribute to a rapid progression to death in patients with acute and lymphoma-type ATL. Immunocompromise is not due to the type of immune ablation observed for HIV-1, even though CD4 cells are infected by HTLV-1; rather, the

TABLE 3 HTLV-associated diseases[a]

Disease	HTLV-1	HTLV-2
Childhood		
Infective dermatitis	++++	No
Persistent lymphadenopathy	++	++
Adult		
ATL	++++	No
HTLV-associated myelopathy	++++	+++
Infective dermatitis	+++	No
Polymyositis	++	Unknown
Uveitis	+++	Unknown
HTLV-associated arthritis	+++	Unknown
Sjögren syndrome	++	Unknown
Strongyloidiasis	++	Unknown
Pulmonary infiltrative pneumonitis	++	++

[a]++++, very strong evidence; +++, strong evidence; ++, possible association; +, weak association; no, evidence does not support association; unknown, no data to support association or lack of association.

ATL, adult T-cell lymphoma.

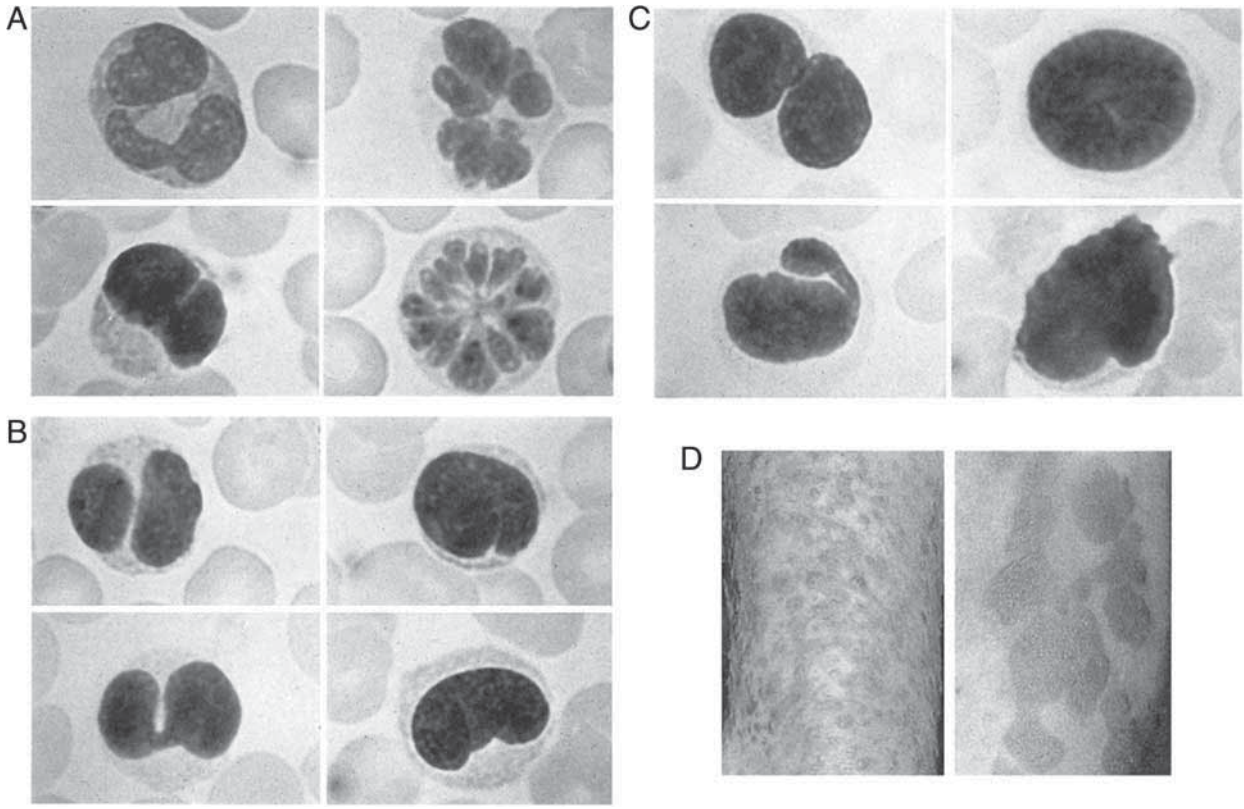

FIGURE 8 Photomicrographs of typical peripheral blood leukemic cells from patients with acute ATL (A), chronic ATL (B), smoldering ATL (C), and cutaneous manifestations (D). Details of the features are discussed in the text.

immunodeficiency is associated with rapidly proliferating malignancy, and the pattern of opportunistic infections is typical for those reported for patients with aggressive non-Hodgkin's lymphomas.

The diagnosis of ATL is based on the demonstration of monoclonal integration of HTLV-1 in tumor tissue, or if molecular diagnostics are not available, presumptive diagnosis can be based upon the presence of HTLV-1 antibodies in a patient with the characteristic clinical features of adult T-cell lymphoma. Proviral HTLV-1 can also be detected in the blood leukemia cells or in biopsy specimens from the patient, but such studies require a laboratory with specialized expertise. In some cases of ATL in patients from high-risk areas with typical clinical features, the HTLV-1 antibodies are absent, but a defective integrated virus with a retained *tax* function can be detected with sophisticated molecular probes.

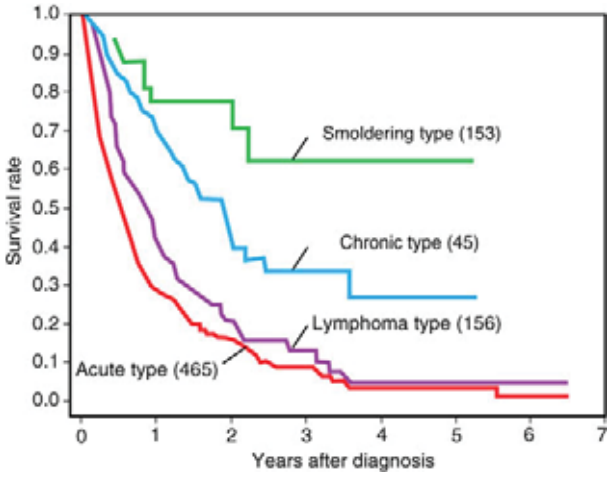

FIGURE 9 Survival patterns of patients with different ATL subtypes after polychemotherapy. Patients with acute and lymphoma-type ATL have the poorest prognosis after chemotherapy. (Adapted from reference 143.)

Other Cancers

HTLV-1 has been detected in isolated cases of other malignancies. In one case of small cell cancer of the lung, viral sequences were monoclonally integrated into the tumor cells (153). There is a statistically increased prevalence of HTLV-1 antibodies in patients with invasive carcinoma of the cervix, but this could result from shared sexual risk factors rather than a direct effect of HTLV-1 in carcinogenesis. Japanese patients with a variety of malignancies show elevated rates of HTLV-1 infections compared with healthy populations, but biases such as blood transfusion might have influenced the association. On the other hand, one study found that HTLV-1 infection was associated with the reduced risk of *H. pylori* carriage and reduced the incidence of stomach cancer (154).

HAM

The onset of HAM is often subtle, and the full clinical picture is not usually seen at first presentation. A single symptom or physical sign may be the only evidence of early

HAM. Symptoms often begin with urinary urgency and frequency and a stiff gait, progressing (usually slowly) to increasing spasticity and lower-extremity weakness, back pain, urinary incontinence, and impotence in men. Patients may complain of sensory symptoms such as tingling, pins and needles, and burning. The vibration sense is frequently impaired. Hyperreflexia of lower limbs, often with clonus and Babinski's sign, may be detected. Hyperreflexia of upper limbs, as well as positive Hoffmann's and Tromner's signs, are also frequent. Exaggerated jaw jerk is seen in some patients, and ataxia sometimes develops. Magnetic resonance imaging shows atrophy of the thoracic spinal cord but, in contrast to multiple sclerosis, only rarely shows cerebral lesions (155). The syndrome differs significantly from classic multiple sclerosis. HAM is usually chronically progressive, without the waxing and waning of symptoms characteristic of multiple sclerosis. Cognitive and cranial nerve findings are not present, and magnetic resonance imaging abnormalities are rare.

HAM patients characteristically have HTLV-1 antibodies or antigens in the blood and the CSF, and HTLV-1 provirus is found in the CSF (156). The CSF may also show a mild lymphocytic pleocytosis; lobulated lymphocytes with morphological similarity to ATL cells may also be present in the blood and the CSF. Mild-to-moderate increases in protein levels may be observed in the CSF, and oligoclonal bonds with specific reactivity to HTLV-1 antigens are detected (122). The definite diagnosis of HAM requires a demonstration of HTLV-1 infection and the exclusion of other causes of myelopathy, such as spinal cord compression, paraneoplastic syndromes, parasitic myelopathy, vitamin B_{12} and folate deficiency, multiple sclerosis, and amyotrophic lateral sclerosis, among others.

The link of HTLV-2 with HAM is now well established (157). Prospective follow-up of a cohort of former US blood donors found a cumulative incidence of HTLV-2 HAM of about 1%, lower than the incidence of HAM among HTLV-1 carriers in the same study (158). CSF lymphocytosis and the detection of HTLV-2 provirus in the CSF were less common than for HTLV-1 HAM, probably due to lower systemic PVLs (158). Earlier reports of HTLV-2 HAM had features reminiscent of the ataxic form of HAM found in Jamaica (159).

Other Syndromes

In areas where the virus is endemic, cases of polymyositis have been associated with HTLV-1 seropositivity (160). A large-joint polyarthropathy has been reported in Japan among elderly patients (147, 161). A distinguishing feature of these cases is the presence of HTLV-1-producing cells in the synovial infiltrate. Another disease associated with HTLV-1 is uveitis. Patients with HTLV-1-positive uveitis complain of blurred, foggy vision or ocular floaters with an acute or subacute onset. Visual acuity is moderately affected. The most characteristic findings are vitreous opacities associated with mild iritis and mild retinal vasculitis. The clinical course is slowly progressive or persistent unless the condition is treated with corticosteroids. More than 90% of cases recur within 3 years, with a mean interval between episodes of 16 months (162).

Disseminated strongyloidiasis refractory to treatment has also been reported to occur in HTLV-1 carriers (163). An increased prevalence of HTLV-1 among tuberculosis patients and a high prevalence of tuberculosis among HTLV-1-infected individuals exist (164). A Japanese study reported a reduced delayed-type hypersensitivity response to *Mycobacterium tuberculosis* purified protein derivative, although a similar study in US HTLV-1 and HTLV-2 carriers showed an intact response to recall antigens (165, 166). Therefore, immune deficits to bacteria and parasites, if they exist among healthy HTLV carriers, are likely to be limited to specific infections.

The association of infective dermatitis syndrome with HTLV-1 infection in children, originally described in Jamaica (167) but also found in other low- and middle-income areas including Brazil and Africa, appears to be an immunodeficiency syndrome induced by HTLV-1 and represents the first childhood HTLV-1 syndrome. These patients are born to HTLV-1-positive mothers and experience a syndrome of failure to thrive. They are prone to refractory generalized eczema with exudates and crusting on the scalp, ear, eyelid margins, paranasal skin, neck, axilla, and groin. Recurrent bacterial superinfections with beta-hemolytic streptococci, *Staphylococcus aureus*, or both are frequent but can be suppressed by chronic antibiotic therapy (168). This syndrome usually emerges in the first few years after birth and can persist into adulthood. Infrequent cases emerging in adolescence suggest that the induction of disease can occur following HTLV-1 infection of young adults. Some affected individuals go on to develop ATL or HAM.

Whether coinfection with HTLV-1 and HIV-1 results in a more rapid progression to AIDS is controversial. Given the effect of HTLV-1 Tax on cellular proliferation, a number of *in vitro* studies document the accelerated killing of HIV-1-infected cells that might predict an influence of HTLV infection on HIV-1-related AIDS progression. Epidemiological studies report varying effects of HTLV-1 infection on accelerating the clinical progression to AIDS, but other studies have not confirmed this observation (169); a recent review has concluded that there is little evidence that HTLV-1 hastens progression to AIDS in coinfected patients (170). However, CD4 lymphocyte counts are elevated in HTLV-1 and HIV coinfection compared to HIV monoinfection, and this needs to be considered when managing the HIV disease.

HTLV-2 Disease Associations

The original isolations of HTLV-2 came from patients diagnosed with T-cell hairy cell leukemia. In one of these cases, the tumor involved B cells, while the HTLV-2 was in the T cells (171). Several cases of large granulocytic cell leukemia (a non-T-cell malignancy with a similar pattern) have also been reported, although larger studies have failed to confirm an epidemiologic association between HTLV-2 and this malignancy (172–174). In addition to HTLV-2-associated HAM (described above), HTLV-2-infected individuals may show increased incidences of acute bronchitis, bladder or kidney infection, arthritis, and asthma and a higher incidence of pneumonia than HTLV-seronegative individuals (136). Finally, a prospective follow-up of the same cohort found increased overall mortality as well as cancer mortality (175). The biological basis for these clinic-epidemiologic findings has not been confirmed but may be due to chronic low-grade inflammation associated with HTLV-2 infection. HTLV-2 does not appear to alter the course of HIV disease in coinfected patients (176).

Disease Incidence

The incubation period between infection and disease onset ranges from months to years. Blood transfusion-associated cases of HAM have developed within a few months of transmission. For ATL, the incubation period appears to be

years to decades. For infective dermatitis, a childhood immunodeficiency condition, disease occurs within the first few months of life following infection at birth or via breast milk transmission.

ATL

In areas of endemicity, such as southern Japan and the Caribbean Islands, the annual incidence of virus-associated leukemia is approximately 3 per 100,000 per year and may account for one-half of all adult lymphoid malignancies (177). ATL occurs in 1 per 1,000 carriers per year, resulting in 2,500 to 3,000 cases per year worldwide (178, 179). ATL rarely occurs in the pediatric age group, but cases in 5- and 6-year-old children have been reported (180). Most ATL cases occur between the late 30s and the late 50s rather than in the older age groups, which is typical of B-cell lymphomas in developed countries. Before the age of 50, HTLV-1 is the major single cause of lymphoma in areas where infection is endemic. Compared to Japan, where the peak occurrence is between 50 and 60 years of age, cases in the West Indies and Brazil among persons of African descent peak approximately a decade earlier; immigrants from these regions to areas where infection is not endemic sustain this differential in the age-specific incidence (50). The male-to-female ratio for ATL cases is approximately 1, which contrasts with the excess of infections among females in adulthood. The decline in ATL incidence after age 50 suggests that early life exposure to HTLV-1 contributes substantially to a subsequent risk of lymphoid malignancy with a latency of decades.

HAM

The lifetime incidence of HAM in HTLV-1 carriers is estimated to be about 2% (range, 0.3% to 4%). The disease clusters geographically in regions of HTLV-1 endemicity. HAM appears to have a shorter latency than ATL and is associated with viral acquisition by sexual intercourse or blood transfusion rather than mother-to-child transmission. HAM cases may be more prevalent because of the long survival associated with this chronic degenerative neurologic condition. Females are approximately twice as likely to be affected with HAM as males; markers of sexual transmission have been associated with the disease in females (181, 182). Cases tend to peak in the 30- to 50-year age group, but cases have been reported for children as young as 3 years of age. HAM is considered uncommon in children, although case reports have increased in recent years (73, 183). In a report of seven children with infective dermatitis and HAM, the progression of neurologic symptoms was remarkably rapid (183). Infections during adult life are more likely to lead to HAM than to ATL.

TREATMENT

Unlike for HIV-1, there is no proven antiviral therapy for HTLV-1. Hypothetically, some drugs that target HIV-1 reverse transcriptase might have effects against HTLV-1, as shown by *in vitro* studies showing the inhibition of viral growth. However, therapeutic effects have not been systematically evaluated in clinical trials, although anecdotal reports suggest some antitumor effects (see below). In ATL the role of active viral replication is far from clear because the tumor cell harbors many oncogenic mutations in cell regulatory genes, which may not be reversible by treating the virus. HAM, with its high viral load and substantial cell-mediated immune response to HTLV-1, would appear to be a better candidate for antiviral treatment, but therapy that targets the immune response itself or a combination of antiviral and immunomodulator therapies may afford an equally attractive avenue for experimental treatment. An increased PVL is a prognostic marker for HTLV-1-associated disease development. A recent report demonstrated that associating histone deacetylase inhibitors with AZT in nonhuman primates naturally infected with STLV-1 allows a very strong decrease in PVL. Thus, this approach could be envisioned in HTLV-1 patients at risk for developing disease. The opportunity exists to investigate the impact of antiviral therapy on persons coinfected with HTLV-1 or HTLV-2 and HIV-1, and studies are needed to determine if, for example, the viral load of HTLV-1 or HTLV-2 is modulated by anti-HIV-1 therapy (184). However, one report indicated that a high viral load of HTLV-2 protected against HIV-1 disease progression. The mechanism appears to involve the heightened production of C-C chemokines in response to HTLV-2 infection, blocking HIV-1 infection (185). The HIV integrase inhibitor raltegravir also has *in vitro* activity against HTLV integration and may be useful in controlling PVL (186, 187). Further study of antiretroviral therapy in HTLV infection is warranted.

ATL

Despite advances in support and the development of novel treatment agents, the prognosis for patients with ATL remains poor. While response rates, even for the poorest risk categories, are over 50% and complete remissions are achieved in 20% of cases, these responses are short-lived, with relapses within weeks to months (58). Therapeutic approaches tested over the past 2 decades have been associated with modest improvements in response, but more recent strategies offer greater hope (188, 189). With a 70% 5-year survival rate with no therapy and because of complicating infections caused by bone marrow suppression, patients with chronic and smoldering ATL are not treated or are given prednisone with or without cyclophosphamide. The acute and lymphoma types of ATL are aggressive high-grade lymphomas with a generally poor prognosis and historically have 20% remission rates at best with a variety of traditional chemotherapy approaches, but remissions are short-lived, and the mortality rate is high.

Negative prognostic factors include poor performance status at diagnosis, age over 40 years, extensive disease, hypercalcemia, and a high serum lactate dehydrogenase level. Approximately 13% to 15% of patients with such aggressive cases experience a long-term survival (over 2 years), which has been associated with several factors: complete remission, longer time to remission, and total doxorubicin dose. Relapses in these long-term survivors often occur in the CNS and prove refractory to subsequent therapy.

A combination of AZT and interferon has been reported to induce remission and improve survival in cases of acute, chronic, and smoldering ATL; the lymphoma type is less responsive to this regimen (190). The mechanism of action of AZT and interferon is not well understood as HTLV replication is not active in most cases of ATL (143). Experimental approaches that use monoclonal antibodies to IL-2R linked with cell toxins selectively targeted to the leukemic cells are being tested, with some evidence of at least partial responses (191). A study of arsenic trioxide demonstrated responses in patients failing prior chemotherapy but involved a high level of toxicity (192). Newer agents like proteasome inhibitors, retinoids, and angiogenesis inhibitors, as well as cellular immunotherapy, are being evaluated (193, 194).

Combinations of doxorubicin and etoposide have resulted in complete remission rates of 40% (195). The current focus of therapy is the use of allogeneic hematopoietic stem-cell transplantation for patients with ATL following an initial complete or partial response to chemotherapy (196) (197). Mortality during transplant is high, but 5-year survival has exceeded 30% to 35%. One transplant patient showed a reappearance of cells harboring the integration of HTLV-1 previously observed in his leukemia cells, but the patient continues in clinical remission, suggesting a possible reversion to the preleukemic carrier state (198). Additionally, some patients have achieved a second complete remission, including by reduction or cessation of immunosuppression, suggesting a graft-versus-ATL effect (196).

HAM

The treatment of HAM with corticosteroids benefits some patients, particularly when given early in the clinical course or in those with rapidly progressive disease, but side effects limit the chronic use of corticosteroids. Treatment with danazol, an androgenic steroid, has reversed urinary and fecal incontinence in some patients but not the spastic limb disease or the underlying neurologic deficit. Experimental studies with beta interferon (IFN-ß) in doses comparable to those used for multiple sclerosis may be of value because the mechanism of immune pathogenesis may be shared between the two diseases (199). Treatment with IFN-α has shown to be of short-term benefit and appears to decrease provirus load (200). IFN-ß-1a interferon has also been reported to reduce the HTLV-1 mRNA load, but the provirus load remained unchanged, and there was only a slight improvement in motor function (201). However, toxicity limits the acceptance of interferon therapy by many patients.

Given the emerging picture of disease pathogenesis with an inability to control high viral expression, therapy with antiviral drugs would appear to be a promising avenue for research. The combination of two nucleoside analogs, zidovudine and lamivudine (AZT and 3TC), was evaluated in 16 patients with HAM (202), but after a year of follow-up, no significant changes in provirus load and no clinical improvements were observed. The HIV integrase inhibitor raltegravir also has activity against HTLV integration, and a current trial is assessing its effects on PVL and clinical course in HAM patients (203).

PREVENTION

Infection Control Measures

The transmission of HTLV is mediated by live cells and not via cell-free body fluids. For this reason, HTLV-1 is not an easily transmitted virus. Universal precautions like those recommended for HIV-1 are applicable for viral inactivation and protection from potentially infectious blood or bodily secretions. Guidelines for prevention and counseling have been developed for HTLV-1 and HTLV-2 by a Centers for Disease Control and Prevention working group (204). Standard prevention approaches address each of the major avenues of transmission and are similar for both viruses: screen blood, eliminate breast-feeding by known infected mothers (or, where not feasible, limit breast-feeding to the first 6 months of life), and advise the use of condoms by discordant couples.

The value of blood donor screening has been well documented in Japan, where up to 15% of HTLV-1 infections have been eliminated (205). In areas where the infection is not endemic, such as the United States and Europe, the cost-effectiveness of screening has been questioned. Due to the low incidence of new HTLV infections, a strategy of antibody testing only first-time blood donors may be cost-effective. Filter-based leukoreduction has been shown to reduce the HTLV-1 PVL in blood units and reduce the risk of transmission, so some countries with low HTLV prevalence have used this approach without testing for virus (83). Reports on HTLV RNA detected in cell-free plasma have not been replicated (206).

Pregnant women in developed countries who are HTLV-1 positive should not breast-feed their infants. However, in developing countries, where safe alternatives to breast-feeding may not be available, limiting breast-feeding to the first 6 months may afford some protection via maternal antibodies (75).

The use of condoms is recommended for couples who are discordant for HTLV infection. Given the likely low frequency of sexual transmission for each sexual encounter, couples who desire a pregnancy could plan to have unprotected sex during periods of maximal fertility. Artificial insemination after washing semen to remove HTLV-infected lymphocytes has been performed. Such decisions require careful discussions between the physician and the patient; reproductive medicine guidelines include HTLV only in some states.

Counseling seropositive patients involves a clear discussion of the distinction of HTLV from HIV. In addition, the HTLV type should be defined by serologic methods, and the distinctions in disease associations of the two virus types should be emphasized. In areas where HTLV-1 is endemic, populations at risk for HIV are also at risk for HTLV-1 (e.g., persons at risk for STDs, persons with high rates of partner exchange, and commercial sex workers), and therefore HIV prevention guidelines will also benefit those at risk for HTLV-1. Prevention measures that promote using condoms, treating STDs, and decreasing high-risk exposures will also prevent HTLV-1 infection. Nosocomial infection has been reported only rarely (89–91), suggesting that HTLV-1 is unlikely to be transmitted in this setting.

There is no therapy for asymptomatic HTLV-1 infection and thus no chemoprophylaxis. Passive immunoprophylaxis is hypothetically effective, as noted below, in animal studies, but it has no practical clinical application given the low risk for transmission except through sexual, breast-feeding, and transfusion exposure, where other prevention methods are more applicable.

Vaccines

While vaccines against HTLV-1 are theoretically feasible, there has been little impetus to develop or market an HTLV-1 vaccine because of the relatively low penetrance of illness and the "orphan disease" status of HTLV-1. Experimentally, vaccines containing whole virus and recombinant HTLV-1 envelope antigens successfully prevent HTLV-1 infection in monkey and rabbit models (207, 208). Protection is correlated with the presence of neutralizing antibodies, indicating that humoral immunity can be an effective barrier against infection, even when the challenge is cell associated. The HTLV-1 envelope is relatively highly conserved, and neutralizing antibody appears to protect against challenge with even major strain variants, consistent with the conclusion that a single serotype will protect against all variants (209). Therefore, a synthetic vaccine against one HTLV-1 isolate could protect against other HTLV-1 isolates. A vaccine that induces cell-mediated immune responses in

non-human-primate studies has also been shown to be effective (210). Therapeutic vaccines include one with Tax peptide-pulsed dendritic cells for adult T-cell leukemia/lymphoma (211) and another based upon Tax and HBZ sequences linked to a lentiviral vector. A preventive vaccine based upon HTLV structural peptides expressed in baculovirus is also being developed (212).

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Human Immunodeficiency Virus

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Infection with human immunodeficiency virus type 1 (HIV-1) is prevalent throughout the world and is characterized by a progressive deterioration of the immune system that is usually fatal if untreated. As of 2013, HIV-1 was estimated to infect 35 million people worldwide (http://www.who.int/hiv/data/en/). Over 95% of these infections are in low- and middle-income countries among young adults. The acquired immunodeficiency syndrome (AIDS) that results from chronic HIV-1 infection is the sixth leading cause of mortality worldwide; it was estimated to have caused 1.5 million deaths in 2013 (http://www.who.int/hiv/data/en/).

Although the first known human case of HIV infection dates to 1959 (1), AIDS was first recognized as a clinical entity in 1981. The syndrome was identified by clusters of unusual diseases including Kaposi's sarcoma and *Pneumocystis jiroveci* pneumonia in young homosexual men who had immunodeficiency due to depletion of $CD4^+$, helper T cells (2–4). AIDS cases were subsequently reported in intravenous drug users (IVDU), hemophiliacs, and in infants born to mothers with AIDS, suggesting a blood-borne as well as sexually-transmitted pathogen. In 1983, HIV-1 was isolated (5), and this novel human retrovirus was proposed as the cause of AIDS. Within several years an antibody test was developed to detect infection, the nucleotide sequence of the genome of HIV-1 was determined (6–9), and the first antiretroviral drug—the nucleoside analogue zidovudine—was shown to have activity *in vitro* and in patients (10, 11). Since then, enormous progress in both basic and clinical research has provided the tools to suppress viral replication to a degree sufficient to prevent or reverse the immunological and clinical sequelae of HIV infection. Nevertheless, neither curative therapy for this disease nor a protective vaccine is available.

Each of the two types of HIV, HIV-1 and HIV-2, reflects the cross-species transmission of nonhuman primate lentiviruses to humans (12). Evidence for cross-species transmission emerged in 1985, when antibodies that were more highly reactive with proteins of simian immunodeficiency virus (SIV) than with those of HIV-1 were detected in West African prostitutes (13). This observation led to the discovery of HIV-2 (14), a virus closely related genetically to SIVsm strains isolated from sooty mangabeys (*Cercocebus atys*). Eight independent transmission events from sooty mangabeys have yielded the HIV-2 genetic groups, A-H, and a ninth unrelated strain was recently identified in Côte d'Ivoire; however, this strain, as well as groups G and H, have been identified only in single individuals, suggesting that human-to-human transmission might sometimes be nonsustained (15, 16). In contrast to HIV-2, the zoonotic sources of HIV-1 are the chimpanzee subspecies *Pan troglodytes troglodytes* (*P.t.t.*), in which SIVcpz strains are endemic, and, to a lesser extent, gorillas, which harbor SIVgor (17–19). Based on genetic analyses, SIVcpz has been introduced into the human population from *P. t. t.* on two occasions to yield the HIV-1 groups M (main; the group responsible for the global pandemic) and N, whereas SIVgor is the source of HIV-1 groups O and P (19, 20). Each of the distinct cross-species transmission events that led to the appearance of the HIV-1 groups (M, N, O, and P) in humans appears to have occurred in southern Camaroon (19).

VIROLOGY

Classification

HIV-1 and HIV-2 are enveloped RNA viruses belonging to the family *Retroviridae*. These viruses reverse transcribe their genomes to form double-stranded DNA, which integrates into the host genomic DNA. HIV-1, HIV-2, and the SIVs are members of the lentivirus genus. Viruses in this genus are characterized by cytopathicity *in vitro*, lack of oncogenicity, the establishment of chronic infections, and relatively slow rates of pathogenesis. SIVs have been found in at least 26 species of nonhuman African primates, including African green monkeys (SIVagm) and sooty mangabeys (SIVsm) (12). In their natural hosts these viruses are generally not pathogenic. However, introduction of the African SIVsm or SIVagm into Asian rhesus macaques results in an AIDS-like illness, similar to that caused by the introduction of SIVcpz into humans (21).

Genotypes, Serotypes, Antigenicity

Among the four genetic groups of HIV-1 (M, N, O, and P), group M viruses dominate the pandemic. This group is further divided into genetic subtypes or clades (A through D, F through H, J, and K) based on nucleotide sequence differences of more than 15% in *gag* and >20% in *env*

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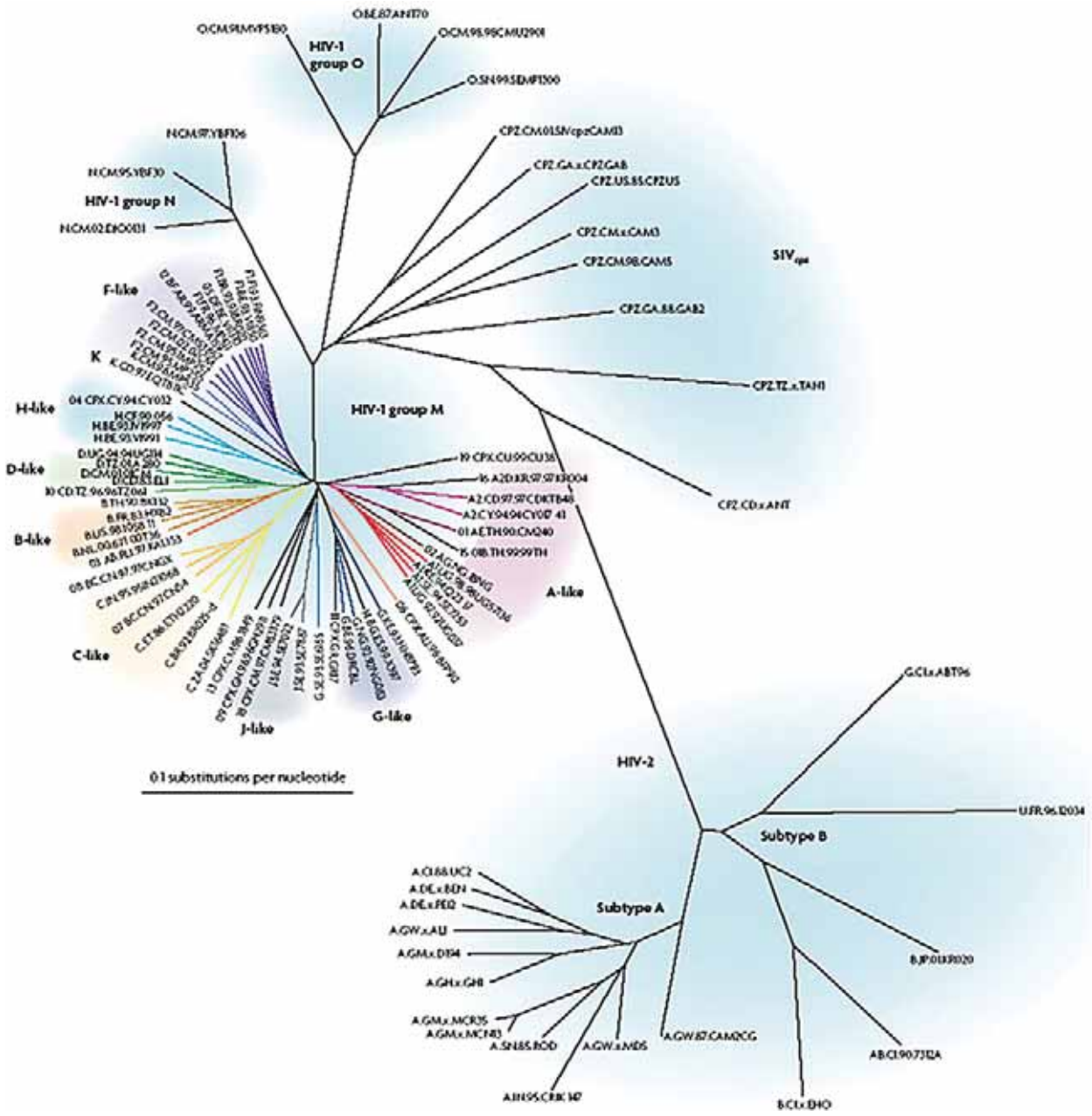

FIGURE 1 The genetic relatedness of different HIV and simian immunodeficiency virus (SIV) strains. 87 human and simian lentiviruses were compared by aligning their full-genome sequences. Phylogenetic trees based on nucleotide distance were constructed by neighbor-joining methods. HIV-2 and HIV-1 share only 50 to 60% sequence identity and cluster at distinct locations on the phylogenetic tree, whereas SIV_{cpz} branches out from the root of the HIV-1 groups. The origins of these HIV-1 groups in southern Cameroon indicate two probable jumps from chimpanzee (groups M and N) and gorilla (group O) species. HIV-1 M subtypes probably evolved from a discrete introduction into the human population and then diverged into different subtypes. The subtypes defined as "A-like" describe HIV-1 isolates with sequences that map phylogenetically more to subtype A than to any other subtype. For example, the recombinant form CRF02_AG (such as 02 AG.NG.IBNG in the HIV-1 group M A-like cluster) has longer genomic segments that are more related to subtype A than to subtype G. M, main; N, new. (Modified with permission from [482]).

(Fig. 1). Subtype C is the most prevalent worldwide. Many viral isolates are recombinants containing sequences from more than one subtype. These are designated CRF for circulating recombinant form (22). For example, the former subtype E is believed to be a recombinant between subtypes A and E, and its current designation is CRF01-AE. Mosaic viruses that contain parts resembling four or more subtypes are given the suffix cpx for complex; for example, the former subtype I has been given the designation CRF04-cpx to indicate that it is a complex circulating recombinant form. The designations of specific HIV-1 strains as CRFs versus parental subtypes are subject to change, since putative evolutionary relationships can be confounded by sampling history and high rates of recombination (23).

Both humoral and cellular immune responses have been detected to each of the proteins of HIV. Antibody reactivity to multiple proteins forms the basis for the confirmatory western blot assay. The primary structural proteins, Gag and Env, elicit the greatest antibody responses, which form the basis for the diagnostic ELISA assay. Neutralizing antibodies are directed to the envelope glycoproteins, gp 120 and gp41, which are exposed on the surfaces of infected cells and virions. However, there are no clearly defined HIV-1 serotypes. The HIV-1 Env is composed of a surface domain (gp120) and a transmembrane domain (gp41), which are noncovalently associated and form trimeric spikes (Fig. 2). The peptide sequence of gp120 contains conserved (C) and variable (V) regions.

Several features of the envelope glycoprotein render the virus relatively resistant to humoral immunity (24, 25). Regions of gp120 that are involved in the binding interactions with cellular molecules required for infectivity are conserved but are poorly accessible to antibodies. For example, the binding site for CD4, the primary cellular receptor for the primate immunodeficiency viruses, is recessed and surrounded by variable, glycosylated regions (Fig. 2). In addition, the surface of gp120 is heavily glycosylated. This glycosylation reduces the antigenicity of Env, partly by allowing the molecule to appear to the immune system as "self." Extensive glycosylation also provides a "shield" that appears to protect virions from neutralizing antibodies. Similarly, the binding site for the chemokine receptors, the so-called coreceptors for these viruses, is masked by the variable loops V2 and V3 (Fig. 2) (26).

Antibodies to gp120 become detectable in the sera of HIV-infected individuals within 2 to 3 weeks after infection (27). However, these early antibodies are not neutralizing. They appear to recognize the interactive regions of gp120 and gp41, which, although immunogenic, are not exposed in the assembled, trimeric, glycoprotein complex (25). Subsequently, antibodies appear that may recognize the V3 or V2 loops and are neutralizing but usually restricted in activity to the infecting strain. Such antibodies appear to drive the selection of escape variants, so that patient serum can neutralize previous, but not contemporaneous autologous isolates (28). Finally, over time, antibodies appear that have more broadly neutralizing activity against a variety of isolates. Many of these interfere with the binding interaction between gp120 and CD4. These antibodies recognize key residues located within the binding pocket for CD4 on gp120 (the so-called CD4BS epitopes; Fig. 2). Other broadly neutralizing antibodies recognize epitopes that are near conserved residues involved in coreceptors binding (the so-called CD4i epitopes; Fig. 2). An unusual neutralization epitope is distant from the receptor binding sites on the outer domain of gp120. This epitope (2G12; Fig. 2) is carbohydrate-based and formed by a conserved cluster of oligomannoses (29). In addition to these epitopes on gp120, broadly cross-reactive neutralization epitopes are also located in the membrane proximal region of the ectodomain of gp41 (30). The last several years have witnessed the isolation of numerous human monoclonal antibodies that are broadly neutralizing; some of their locations on the envelope glycoprotein trimer are shown in Fig. 2.

Composition of Virus

Virion Morphology, Structure, Size, Genomic Organization

By electron microscopy, the HIV-1 virion measures approximately 100 to 150 nm in diameter (Fig. 3). Mature viral particles are characterized by an electron-dense, conical

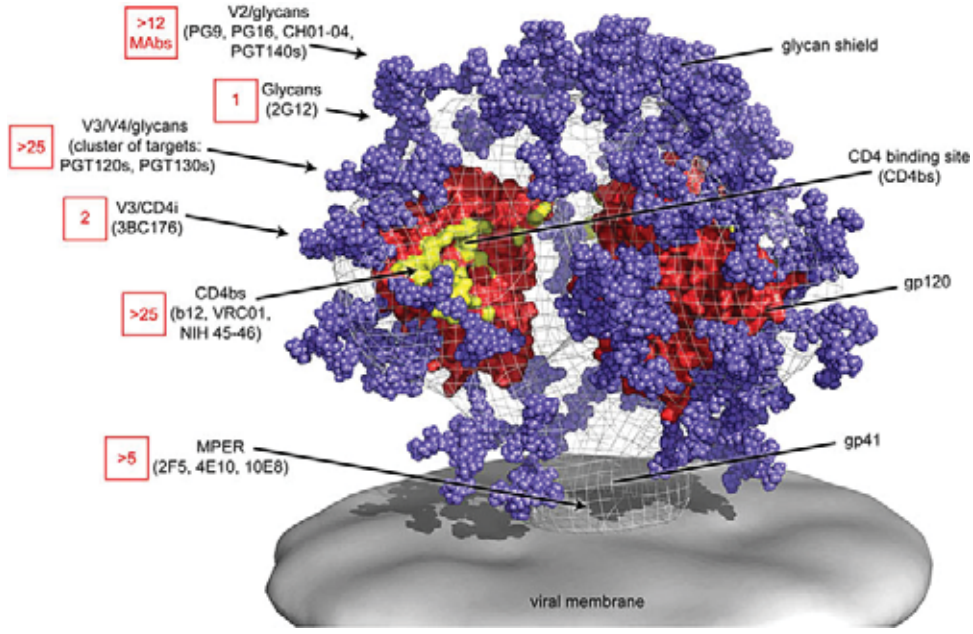

FIGURE 2 Structure and antibody recognition of the HIV envelope spike. The molecule is a heterotrimer of composition (gp120)3 (gp41)3. Gp41 is a transmembrane protein and gp120 is the receptor molecule for CD4 and CCR5 (or CXCR4). The model is adapted from a cryo-electron tomographic structure of the HIV trimer, with the crystal structure of the b12-antibody-bound monomeric gp120 core (red) fitted into the electron density map. Glycans are shown in purple. The CD4 binding site is shown in yellow. The approximate locations of the epitopes targeted by existing broadly-neutralizing monoclonal antibodies (bnMAbs) are indicated with arrows, and the number of MAbs targeting each epitope is shown in red boxes. A small selection of bnMAbs targeting each epitope is included. (Modified with permission from [483]).

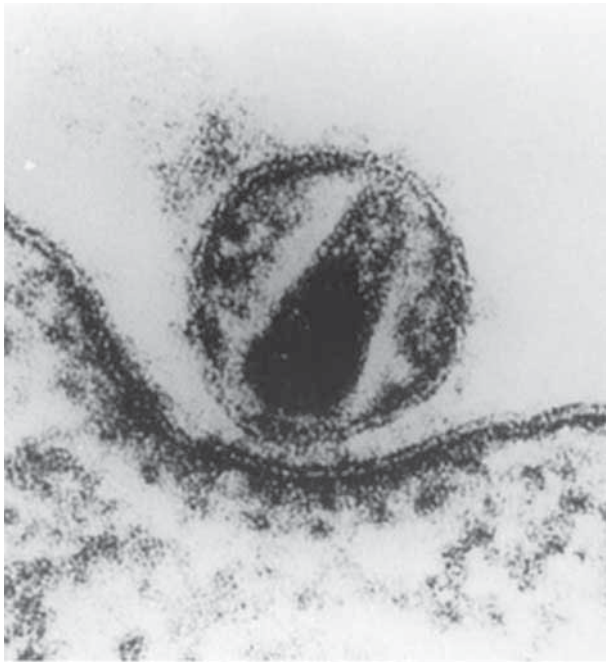

FIGURE 3 Electron micrograph of an HIV-1 virion budding from an infected cell. The viral glycoprotein complexes are barely discernable on the surface of the virion membrane. The electron-dense material just beneath the viral lipid bilayer corresponds to the MA (p17) protein. The conical virion core is composed of the CA (p24) protein. Also with the core are the NC (p7) protein, which binds the genomic RNA, the p6 protein required for budding, the accessory protein Vpr, the reverse transcriptase, the integrase, and two copies of the genomic RNA. The accessory-protein Nef is also virion-associated. Micrograph courtesy of H. Gelderbloom.

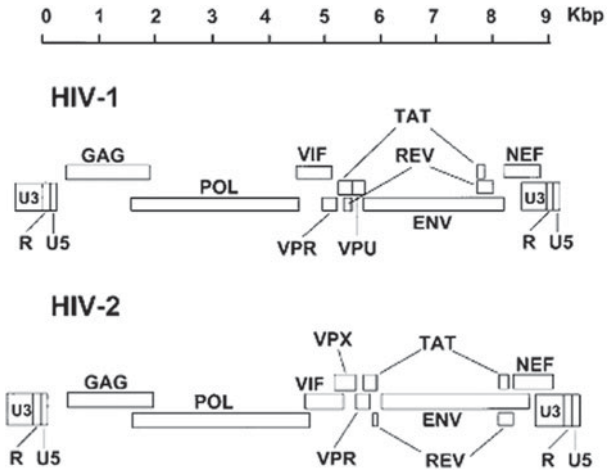

FIGURE 4 Genomic organization of HIV-1 and HIV-2. The viral open reading frames (ORFs) are shown; the roles of their gene products are described in the text and in Table 1. The ORFs of the *tat* and *rev* genes are interrupted by an intron. The long terminal repeats (LTRs), found at each end of the fully reverse-transcribed viral DNA, are composed of U3, R, and U5 regions; the definitions of their boundaries are described in the text. The major genetic differences between these viruses are the lack of the Vpu ORF and the presence of the Vpx ORF in HIV-2; these features render HIV-2 more similar to most SIVs than to HIV-1.

core. The core is surrounded by a lipid envelope that is acquired as the virion buds from the infected cell. The virion core contains two copies of single-stranded, positive sense, genomic RNA, each of which encodes the complete viral repertoire of structural, enzymatic, and regulatory proteins. The HIV genome is approximately 10kb in length and is organized similarly to other retroviruses (Fig. 4). The sequence is flanked by the two long-terminal repeats (LTRs). The 5′ LTR contains the enhancer/promoter sequences for viral transcription, and the 3′ LTR contains the polyadenylation signal. From 5′ to 3′ the viral genome contains the *gag* gene, which encodes the virion structural components; the *pol* gene, which encodes the viral enzymes; and the *env* gene, which encodes the envelope glycoproteins. The primate immunodeficiency viral genomes contain six additional genes: *vif*, *vpr*, *tat*, *rev*, *nef*, *and vpx* in the case of HIV-2 and SIV, or *vpu* in the case of HIV-1 (Fig. 4 and Table 1) (31). The products of these genes function via interactions with host cell proteins to optimize viral replication by a variety of mechanisms discussed below.

Major Structural and Regulatory Proteins

The major structural and core proteins of HIV are synthesized from *gag* as a large, myristoylated precursor polyprotein (pr55) that is subsequently cleaved by the viral protease to yield the matrix (MA; p17), capsid (CA; p24), and nucleocapsid proteins (NC; p7) (32, 33). The matrix protein is a peripheral membrane protein located along the inner leaflet of the viral lipid envelope, where it directs the incorporation of the envelope glycoproteins (Env) into the forming virion (34). The capsid protein (p24) assembles to form the conical core of the virion. The core structure follows the principles of a fullerene cone composed primarily of a curved array of hexameric CA subunits, with the inclusion of several pentameric subunits to allow closure of the cone (35). The nucleocapsid protein (p7) is an RNA binding protein required for packaging of the genomic RNA into the virion (36). Several smaller cleavage products, p1, p2, and p6, are also generated from the p55 precursor. The p6 protein contains the so-called "late" or "L" domain required for viral budding; these domains mediate interactions with cellular proteins of the ESCRT (endosomal sorting complexes required for transport) complex to mediate membrane scission during the budding process (37). The p6 protein also mediates the incorporation of the viral accessory protein Vpr into virions (38).

The viral enzymes are also produced by proteolytic cleavage from a large precursor polypeptide. During translation, a ribosomal frameshift occasionally occurs between the *gag* and *pol* ORFs, resulting in the synthesis of a *gag-pol* precursor protein (pr170) (39). The ratio of Gag-Pol to Gag produced in infected cells is approximately 1:20. Subsequent cleavage of pr170 yields the Gag protein products and the retroviral protease (p11), reverse transcriptase/ribonuclease H (p66/p51), and integrase (p32). The aspartyl protease p11 cleaves the structural and enzymatic proteins from the large polyprotein precursors. This enzyme is a symmetric dimer that is activated by dimerization during virion assembly (39, 40). Pharmacologic inhibition of the viral protease results in the formation of noninfectious particles. The reverse transcriptase enzyme is a heterodimer with 66 kDa- and 51 kDa-subunits. This enzyme provides both the reverse transcriptase activity, which allows RNA-dependent DNA polymerization, and the ribonuclease H activity, which specifically degrades RNA present in RNA/DNA heteroduplexes. The RNase H

TABLE 1 HIV genes and gene products

Gene	Protein	Size	Function
Structural			
gag	Matrix (MA)	p17	Structural protein (recruits envelope glycoprotein)
	Capsid (CA)	p24	Structural protein (forms conical core)
	Nucleocapsid (NC)	p7	Binds viral RNA to encapsidate genome
	p6	p6	Budding
pol	Protease (PR)	p12	Viral enzyme: cleavage of polyprotein precursors
	Reverse transcriptase (RT)	p66/p51	Viral enzyme: reverse transcription and RNase H activities
	Integrase (IN)	p32	Viral enzyme: integration of viral cDNA into host chromosomes
env	Surface glycoprotein (SU)	gp120	Viral envelope glycoprotein: receptor binding
	Transmembrane (TM)	gp41	Viral envelope glycoprotein: fusion
Regulatory/ Immune Evasion			
tat	Tat	p14	Transactivates viral transcription
rev	Rev	p19	Transports unspliced mRNA to cytoplasm
vif	Vif	p24	Promotes virion infectivity by degrading cellular cytidine deaminases in the APOBEC3 family
nef	Nef	p27	Down-regulates class I MHC and CD4; enhances viral infectivity; facilitates T-cell activation
vpu[a]	Vpu	p16	Promotes release of viral particles by counteracting BST2/tetherin; induces degradation of CD4
vpr	Vpr	p15	Arrests cell cycle in G_2/M; facilitates viral replication in macrophages
vpx[a]	Vpx	p14	Facilitates viral replication in dendritic cells and macrophages by degrading the cellular enzyme SAMHD1

[a]*vpu* is found almost exclusively in HIV-1; *vpx* is found only in HIV-2 and SIV.

domain is present only in the larger of the two subunits (41). The integrase mediates insertion of the viral DNA into that of the chromosome of the host cell (42). To accomplish this, integrase comprises two activities: a DNA cleaving activity, which cuts the host DNA and processes the ends of the HIV-DNA, and a DNA strand-transfer activity which covalently attaches the viral DNA to that of the host, most frequently in transcriptionally-active ORFs (43).

The protein product of the *env* gene is synthesized in the endoplasmic reticulum as an 88 kDa polypeptide (44). This protein undergoes glycosylation in the endoplasmic reticulum and Golgi. The resulting molecule, gp160, contains N-linked, high-mannose sugars that account for approximately half of the final molecular weight of 160 kDa. Much of the newly-synthesized gp160 is retained in intracellular compartments and is eventually degraded; however, a fraction is cleaved by cellular serine proteases like furin to generate the transmembrane (gp41) and surface (gp120) subunits (44). This cleavage event is required for viral infectivity because it allows for subsequent exposure of a sequence within the ectodomain of gp41 that mediates fusion of the virion and target cell membranes (45). Following proteolytic cleavage, gp120 remains noncovalently associated with gp41 on the cell surface. The complex is incorporated into the envelope of budding virions via an interaction between the cytoplasmic domain of gp41 and the p17 matrix protein (34). The envelope glycoproteins are arranged as trimers in a spike-and-knob configuration (Fig. 2). As few as 8 to 14 Env trimers may be incorporated per virion (46, 47).

The primate immunodeficiency viruses encode several nonenzymatic and nonstructural gene products (31). Two of these genes, *tat* and *rev*, are essential for viral replication. Tat is a 14-kDa *trans*-activating protein that markedly enhances the rate of transcription from the 5′ LTR by recruiting cellular factors that enhance the processivity of the cellular RNA polymerase II complex (48–50). Tat recruits a cyclin-dependent protein kinase complex (Cdk9 plus cyclin T) to a structured region near the 5′ end of the primary viral transcript (the transacting responsive region or TAR) (51, 52). Cdk9 hyperphosphorylates the C-terminal domain of RNA polymerase II, allowing efficient elongation of the nascent viral mRNA (53). In the absence of Tat, viral transcription is essentially stalled just after initiation.

The 18-kDa Rev protein mediates the transport of singly-spliced and unspliced viral RNAs from the nucleus to the cytoplasm. These RNAs encode the HIV-1 structural and enzymatic proteins and include the viral genomic RNA (54). Rev directly links these viral RNAs to a specific nuclear export pathway. The N-terminal region of Rev contains a basic domain that binds a structured region in the RNA (the Rev-response element or RRE, which overlaps the coding sequence of the gp41), while the C-terminal region contains a prototypical, leucine-rich, nuclear export signal that binds to the nuclear export protein CRM1, also known as exportin 1 (55–57). In the absence of Rev activity, viral mRNAs are inefficiently exported from the nucleus, and, as a consequence of prolonged nuclear retention, they undergo extensive splicing to yield subgenomic mRNAs encoding only a subset of viral proteins that includes Tat, Rev, and Nef.

The other nonenzymatic and nonstructural genes of HIV-1 and HIV-2 (*vif, vpr, vpx, vpu,* and *nef*) are not essential for viral replication under certain conditions *in vitro* and consequently have been termed "accessory genes." Nevertheless,

these genes are highly conserved among primate lentiviruses, and their importance has been documented using the macaque/SIV and SHIV animal models of AIDS as well as by the use of relatively physiologic *in vitro* systems, such as primary cultures of T lymphocytes and macrophages (58–62). Moreover, the role of these gene products in providing evasion of host innate and adaptive defenses has become increasingly clear. The accessory gene products function by mediating interactions with cellular proteins to co-opt protein trafficking or degradation pathways. With the exception of Nef, each of these gene products modulates the substrate specificities of cellular ubiquitin-ligase complexes to cause the degradation of cellular proteins that are deleterious to the virus (63).

The 23-kDa Vif protein targets cellular cytidine deaminases, in particular APOBEC3F and APOBEC3G, for degradation by the proteasome via the cullin 5 ubiquitin ligase complex (64, 65). In the absence of Vif, APOBEC proteins are incorporated into virions, where they deaminate cytosine residues in the minus strand of the forming viral cDNA. The resulting uracil residues lead to inactivating hypermutation (G to A transitions in the plus strand) (66). Moreover, some APOBEC proteins inhibit reverse transcription directly (67). Thus, Vif is required for the production of infectious virions (68), and it is required for viral replication in primary cultures of T lymphocytes (58). APOBEC cytidine deaminases appear to provide an innate cellular defense not only against exogenous retroviruses such as HIV-1 but also endogenous retroelements (69).

The 14-kDa HIV-1 Vpr protein has at least two functions. First, Vpr arrests cells in the G2/M phase of the cell cycle (70). Cell-cycle arrest increases the yield of progeny virions due to enhanced viral transcription during the prolonged G2 phase (70), and it is associated with apoptosis (programmed cell death) (71). Second, Vpr facilitates efficient viral replication in macrophages, partly by stabilizing the envelope glycoprotein and enhancing its incorporation into virions (59, 72). These activities require the association of Vpr with a cullen 4-based ubiquitin ligase complex (73). This in turn, prematurely activates a host endonuclease complex, causing the arrest of the cell cycle and inhibiting the ability of the cell to "sense" viral DNA and respond by producing interferon (74). Vpr-activities can occur immediately after infection of the cell (75) because the protein is incorporated into virions by direct association with the p6 *gag* gene product (76).

The 14 kDa Vpx protein, not present in HIV-1, is found in HIV-2 and most SIVs. Vpx is genetically related to Vpr and probably arose from gene duplication. Like Vpr, its activity is mediated through the host cullen 4-based ubiquitin ligase complex. Vpx triggers degradation of the host enzyme, SAMHD1, a triphosphorylase and ribonuclease that restricts the replication of primate lentiviruses in myeloid cells, including dendritic cells and macrophages (77, 78). Thus, Vpx accounts for the differential ability of HIV-2, but not of HIV-1, to replicate efficiently in dendritic cells.

The 16 kDa Vpu protein is an integral membrane protein encoded by HIV-1 but not by HIV-2 or most SIVs (79). Vpu modulates cellular transmembrane proteins. It removes the host protein bone marrow stromal cell antigen 2 (BST2), also known as tetherin, from the plasma membrane. BST2 is an interferon-inducible protein that traps certain enveloped viruses, including HIV-1, on the surface of the infected cell just after budding (80–82). In the absence of Vpu, virions trapped by BST2 not only fail to contribute to cell-free infectivity, but they also increase the density of envelope glycoprotein (Env) antigens on the cell surface and render infected cells more susceptible to killing by antibody-dependent cytotoxicity (ADCC) (83, 84). Vpu induces the degradation of CD4, the primary cellular receptor for the virus, by linking it, while still in the endoplasmic reticulum, to the proteosomal degradation machinery via the cellular protein ß-TrCP and the cullin1 ubiquitin ligase complex (85). By degrading CD4, Vpu prevents the decreased infectivity of progeny virions that would otherwise occur due to the interaction of Env and CD4 (86). Moreover, this activity of Vpu prevents the exposure of CD4-induced epitopes within Env on the cell surface that are especially good targets for ADCC (87, 88). Vpu modulates several other cellular proteins, including NTB-A, a coactivator of natural killer (NK) cells; CD1d, a major histocompatibility complex (MHC)-like molecule involved in the presentation of lipid antigens; and CCR7, a receptor involved in chemotaxis (89–91). Overall, these activities support an overarching role of Vpu in immune evasion.

The 27-kDa Nef protein modulates both cellular signal transduction and membrane trafficking. Nef is a peripheral membrane protein that modulates T-cell activation via its interaction with cellular kinases (92). Nef also directly affects membrane trafficking by interacting with cellular proteins that coat transport vesicles and linking its targets to them (93–96). The consequences of this include the down-regulation of CD4 and class I MHC from the cell surface (97, 98). The down-regulation of class I MHC provides escape from immune surveillance by enabling infected cells to avoid destruction by cytotoxic T lymphocytes (CTL) (99). The down-regulation of CD4 by Nef provides optimal virion infectivity, and, like Vpu, it prevents the exposure of CD4-induced epitopes in Env on the cell surface (87, 88, 100). Nef also enhances virion infectivity in a manner potentially dependent on the stability of Env trimers; this results from antagonism of cellular transmembrane proteins in the serinc family that specifically inhibit the activity of retroviral envelope glycoproteins (101–104).

Biology

Replication Strategy (Fig. 5)

The entire replication cycle of HIV-1, from the binding of virions to target cells to the release of infectious progeny, is completed in approximately 24 hours, *in vitro* and *in vivo* (105, 106). Replication is initiated by attachment of the virus to a target cell through the interaction of the viral envelope glycoprotein, gp120, with the cellular receptor molecule, CD4 (107–109). The binding of gp120 to CD4 induces conformational changes in gp120, which enable binding to the cellular coreceptor molecules (110). The two major coreceptor molecules for HIV-1 are CXCR4 and CCR5 (111–113), transmembrane proteins that function as receptors for chemoattractant cytokines (chemokines). The natural ligand for CXCR4 is the stromal cell-derived factor 1 (SDF-1), and the natural ligands for CCR5 are the ß-chemokines CCL3, CCL4, and CCL5 (formerly known as MIP-1α, MIP-1ß, and RANTES, respectively). Binding of gp120 to these coreceptors is obligatory for the fusion of virus with the host cell, and natural or synthetic ligands for these molecules can block the infectivity of HIV-1. Some primary isolates of HIV-1 can utilize either CXCR4 or CCR5 as a coreceptor for entry, but many can utilize only CCR5. Binding of gp120 to the coreceptors allows exposure of a fusogenic motif in the amino-terminal ectodomain of gp41; which leads to fusion of the lipid bilayer of the virion with

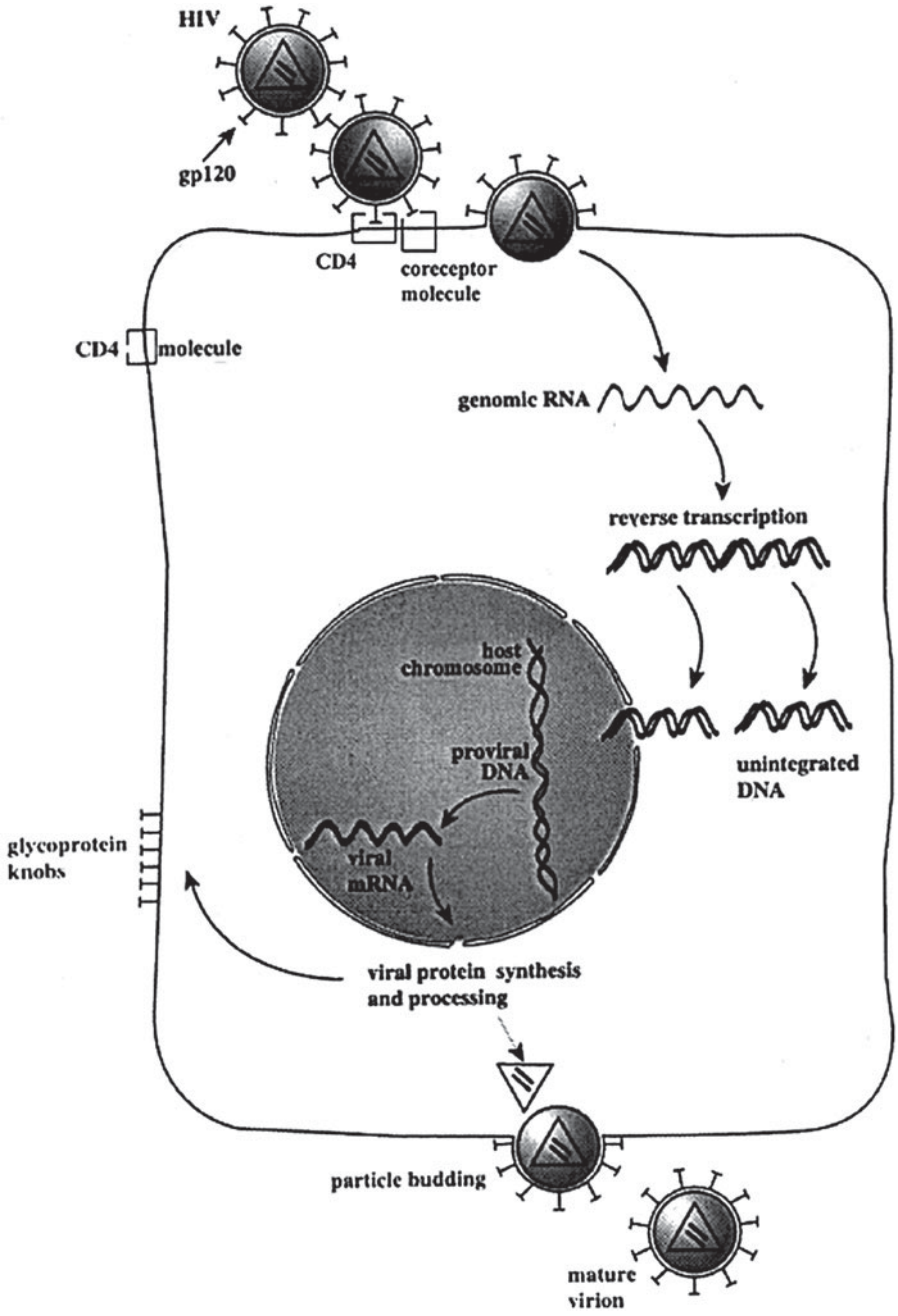

FIGURE 5 Replication cycle of HIV.

that of the host cell (114). The fusion-competent core of the gp41 forms a six-helix bundle analogous in structure to the low-pH (fusion-competent) conformation of the influenza A virus hemagglutinin (115). Formation of this so-called "hairpin" conformation is required for the viral and cellular membranes to reach sufficient proximity to fuse. Entry of HIV into the cell occurs from endosomal as well as plasma membranes, although unlike influenza virus, the process is acid-independent and is not blocked by weak bases or drugs that inhibit acidification of the endo-lysosomal system (116, 117).

Synthesis of the viral cDNA can begin in cell-free virions (118), but it is completed in the target-cell cytoplasm within so-called reverse transcription complexes that include the viral capsid, which travels toward the cell nucleus along microtubules (119). The synthesis of the viral cDNA is discontinuous. The reverse transcriptase (RT) synthesizes the first complementary strand of DNA (the minus strand) using the viral genomic RNA as a template and a host tRNA-lys as a primer. The tRNA primer-binding site defines the 3′ end of the U5 region of the LTR. Viral ribonuclease H degrades the original RNA template. The reverse transcriptase (RT) then synthesizes the second strand of DNA (the plus strand), beginning at two polypurine tracks: one defines the 5′ end of the U3 region of the LTR, and the other is located near the center of the genome (120, 121). Strand-switching events

that utilize sequence complementarity at each end of the viral RNA occur during the synthesis of both minus- and plus-strand DNAs, generating the U3-R-U5 direct repeats (the LTRs) at each end of the complete, double-strand cDNA. These strand-transfer events lead to the utilization of both copies of the virion-associated genomic RNA as templates during reverse transcription, so that genetic recombination occurs when the two copies are dissimilar in sequence (122). Such recombination generates genetic variants following co-infection of the same target cell with genetically distinct viruses.

The reverse-transcribed viral genome remains associated with the capsid until transport into the nucleus, which occurs in nondividing cells through nuclear pores. The capsid itself contains binding sites for several cellular proteins that facilitate this process, including nuclear pore proteins (123). After entry into the nucleus, the double-stranded, linear DNA is integrated into the host-cell chromosome by the virally-encoded integrase in concert with host-cell DNA repair enzymes to form the provirus (124, 125). Covalently-closed DNA circles with either one- or two-LTR junctions are also formed by host enzymes in the nucleus, but these forms are dead-end products that cannot integrate into the target-cell DNA. Integration requires the cellular co-activator protein p75/LEDGF (126), which tethers the viral integrase enzyme to the host-cell chromatin. Chromosomal integration is required for viral replication. Integration also yields the form of the viral genome responsible for establishing a long-lived, latent reservoir in the host.

The activation of HIV transcription and gene expression from the integrated provirus is dependent on the activity of both cellular and viral factors. The virion-associated protein Vpr is a weak transactivator of transcription and may stimulate the initial expression of viral RNAs (75). Specific sites within the 5′ LTR are recognized by cellular transcription factors including NF-κB, AP-1, SP1, and nuclear factor of activated T-cells (NFAT), several of which are induced during the activation of T cells (127). Transcription is initiated from a single site in the viral 5′ LTR, the 5′ boundary of the R region. The activity of the HIV promoter is repressed by chromatin structure; chromatin remodeling induced by histone acetylation can relieve this repression and activate transcription (128). The primary transcript is alternatively spliced to generate over 30 species of viral mRNAs. The early viral transcripts are extensively spliced to form a group of mRNAs that are 1.8 to 2.0 kb in size and which encode the proteins Tat, Rev, and Nef (129). Tat increases the level of viral transcription markedly by recruiting cellular factors to the nascent RNAs, facilitating their elongation as discussed above. Once Rev is synthesized, the incompletely spliced- (approximately 5 kb) and unspliced- (9 kb) viral mRNAs are exported from the nucleus (130). These RNAs encode the accessory gene products Vif, Vpr, and Vpu, all the structural and enzymatic proteins, and the genomic RNA. Thus, Rev mediates a transition from early gene expression (Tat, Rev, and Nef) to late gene expression (Gag, Pol, Env, Vif, Vpr, and Vpu) about six to eight hours after infection *in vitro* (105).

The assembly of virions occurs primarily at the plasma membrane (131). The Gag and Gag-Pol precursor polyproteins are synthesized in the cytoplasm and associate with the plasma membrane via N-terminal myristoylation and binding to specific membrane lipids (132). Dimerization of the Gag precursor activates the viral protease, which cleaves the precursor proteins to generate the individual structural and enzymatic polypeptides described above. In contrast to Gag, the Env glycoproteins are synthesized in association with cellular membranes. Env is then recruited into the forming virion at the plasma membrane by association of the cytoplasmic domain of gp41 with p17 MA. The capsid protein p24 forms the core structure, and the nucleocapsid protein p7 binds the genomic RNA. The Gag polyprotein is sufficient for virion assembly and budding. Budding requires a specific domain (the "L-domain") in the p6 region of Gag, which binds endosomal proteins (the ESCRT complexes) involved in the biogenesis of multivesicular bodies (MVBs) (133, 134). MVBs are late endosomal structures that contain internal vesicles, and the viral budding process is topologically similar to the formation of the vesicles within MVBs. Virion assembly occurs selectively at the basal-lateral surfaces of polarized epithelial cells, at the uropod of T cells, and at regions of cell-cell contact that are enriched in cellular transmembrane proteins termed tetraspanins (135, 136). HIV-1, like measles and influenza virus, buds from specialized regions of the plasma membrane that are enriched in cholesterol and glycolipids (so-called "lipid rafts") (137). This modified lipid composition presumably facilitates budding and/or the fusion of the virions with target cells. In addition to the virally-encoded proteins, virions of HIV-1 incorporate a number of cellular proteins, including major histocompatability antigens (138), adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1) (139), cyclophilin A (140), and several cytoskeletal and endosomal proteins (141). The roles of most of these virion-associated cellular proteins for the virus are not clear, although the incorporation of CD55 and CD59 protect virions from inactivation by complement (142).

Host Range, Tropism, CPE

The natural host range of HIV-1 and HIV-2 is restricted to humans, although chimpanzees can be infected with HIV-1 under experimental conditions (143, 144), and cynomologous monkeys and rhesus macaques have been infected with HIV-2 (145, 146). Unlike infection in humans, HIV-1 is generally nonpathogenic in chimpanzees, and virus replication declines over time.

Studies of the molecular determinants of the host range of primate lentiviruses have revealed novel, innate intracellular defense mechanisms that restrict infection. For example, Old World monkey cells cannot support infection with HIV-1 due to the species-specific activity of the host protein TRIM5α, which targets the capsid protein of incoming virions to inhibit viral replication (147). Similarly, human BST2 presented a barrier to the cross-species transmission of SIVcpz to humans, which was overcome by evolution of the *vpu* gene (148).

The primary cellular targets for HIV-1 infection *in vivo* are $CD4^+$ T lymphocytes and macrophages. *In vitro*, HIV-1 replicates efficiently in primary cultures of $CD4^+$ T cells, while only a subset of isolates are capable of replication in immortalized $CD4^+$ T-cell lines. This cellular tropism is the result of the selective interaction of Env glycoproteins with specific coreceptor molecules (149). Many primary isolates (derived from patients by minimal passage in primary cultures of T cells) utilize CCR5 exclusively and fail to grow in T-cell lines, which typically express CXCR4 but not CCR5. Other primary isolates utilize both CCR5 and CXCR4, and consequently these isolates grow in both primary cells and T-cell lines. Exclusive use of CXCR4 is a feature of occasional clones from later-stage patients and laboratory strains of HIV-1 that have been adapted by extensive passage using CXCR4-expressing immortalized T cells. Macrophage-tropic

isolates utilize CCR5 as the cellular coreceptor. The basis of macrophage tropism remains incompletely understood because, although these cells express CXCR4, they are not permissive for the replication of viruses that use only this coreceptor. Within Env, the principal determinants of specific coreceptor usage and macrophage tropism map to the V3 loop, but other regions of gp120 are also important (150, 151). Macrophages have very low levels of CD4, and the ability of certain Env proteins to enable viral entry into cells expressing such low levels might be the key determinant of macrophage tropism (152). HIV enters dendritic and Langerhans cells via the interaction between gp120 and C-type lectins on the cell surface. Although these professional antigen-presenting cells do not support robust productive infection, they can retain infectious virions and transmit them to $CD4^+$ T cells (153, 154). This so-called transinfection may be an important part of the mechanism of transmission of HIV at mucosal surfaces and of its initial spread to regional lymphoid tissue.

In vitro, the fusion of infected and uninfected cells leads to a cytopathic effect characterized by the formation of multinucleated giant cells (syncytia) (Fig. 6). Not all primary isolates of HIV-1 induce syncytia in immortalized T-cell lines *in vitro*. Isolates derived early in the course of infection are usually nonsyncytium-inducing (NSI), while isolates from later stage patients often, but not always, have a syncytium-inducing (SI) phenotype (155, 156). The NSI phenotype is caused by exclusive use of CCR5 by the Env of the isolate, while the SI phenotype is caused by the additional ability to use CXCR4, allowing viral growth in T-cell lines. In longitudinal analyses of patient isolates, the acquisition of tropism for cells expressing only CXCR4 is associated with more rapid decline in $CD4^+$ T cells and clinical progression of disease (157, 158). The use of CXCR4 enables the virus to infect naïve CD4-positive T cells, whereas the use of CCR5 enables the virus to infect memory CD4-positive T cells (159). Thus, the additional use of CXCR4 as a coreceptor is a marker for expanded tropism including both naïve and memory CD4-positive T cells, potentially explaining the association of this phenotype with more rapidly progressive disease.

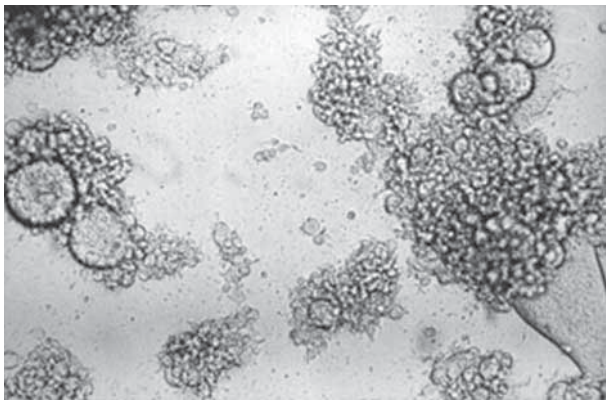

FIGURE 6 Formation of syncytia during replication of HIV-1 in a culture of T lymphoblastoid cells. In immortalized T-cell lines, the interaction of the viral-glycoprotein complex with the cellular receptors CD4 and CXCR4 allows cell-cell fusion and the formation of multinucleated giant cells. The formation of syncytia begins with cell clustering, followed by cell-cell fusion and the ballooning of cell membranes.

Inactivation by Physical and Chemical Agents

HIV is sensitive to a variety of chemical agents including glutaraldehyde, hypochlorite, quaternary ammonium compounds, phenolics, ethanol, and iodine (160). Cell-free virus is inactivated most readily; infectivity is decreased by at least 10^6-fold after 1 minute of exposure of cell-free virus to 0.5% glutaraldehyde or 35% ethanol. Cell-associated virus is less susceptible to inactivation; exposure of cell-associated virus to 35% ethanol for five minutes is inadequate, but exposure to 75% ethanol for one minute is sufficient. Cell-associated virus suspended in blood is the most resistant to inactivation; glutaraldehyde (0.5%), hypochlorite (25,000 ppm), and 75% ethanol are effective after one minute, but quaternary ammonium compounds and phenolics are ineffective even after 10 minutes.

HIV is also sensitive to ultraviolet light (160). As in the case of chemical inactivation, cell-associated virus and virus in blood are more resistant to inactivation by ultraviolet light. A 10-minute exposure of cell-free virus to ultraviolet light in a typical biosafety cabinet is sufficient to inactivate HIV, but 30 minutes is required to inactivate cell-associated virus, and even 60 minutes fails to inactivate cell-associated virus suspended in blood. HIV-1 is inactivated by heating, which is useful not only for the safety of preparations of clotting factors but also for the safety of breast milk from HIV-infected mothers (161, 162).

EPIDEMIOLOGY

Distribution and Geography

The AIDS pandemic can be viewed as a composite of multiple epidemics, each occurring in specific geographic regions and populations. While HIV-2 has remained largely confined to West Africa, HIV-1 has spread throughout the world. Group M viruses are responsible for the vast majority of HIV-1 infections. Distinct subtypes or clades of Group M viruses have been isolated in geographically distinct regions of the world. In the United States, Europe, and Australia, the prevalence of subtype B illustrates a founder effect in which one or several viral variants were introduced and then disseminated through the population (163). Subtype B is found rarely in Africa, whereas subtype A predominates in West Africa, subtype A and D in East Africa, and subtype C in southern Africa. Variants of HIV-1 appear to quickly expand to become the major subtype when introduced into a specific population or geographic area. For example, in Thailand, subtype B viruses predominated among intravenous drug users in Bangkok, while CRF-01AE (formerly designated subtype E) recombinant viruses spread throughout the country by heterosexual contact (164).

The distribution of HIV-1 subtypes is complex, uneven, and probably reflects a stochastic dissemination. In regions like central Africa, Southeast Asia, and South America, where different subtypes are prevalent, the incidence of co-infection and subsequent recombination between subtypes may be high (165). Intersubtype recombination may be high in areas such as Africa and Southeast Asia, where multiple subtypes of HIV-1 are known to circulate simultaneously. For example, in Camaroon, mixed infections were observed in approximately 10% of cases and included co-infection with two subtypes within group M, co-infection with M and O viruses, and even co-infection with HIV-1 and HIV-2 (166). Recombination events have been described between all of the HIV-1 subtypes belonging to group M. Recombination has also been described between Group M and O viruses (167).

Incidence and Prevalence of Infection

According to WHO estimates, approximately 30 million people are estimated to have died, while 35 million are living with HIV infection or AIDS. About 2.5 million new cases were estimated to occur globally during the year 2006. Approximately 95% of all HIV infections have occurred in the developing world and among young and middle-aged adults.

The prevalence and incidence rates of HIV infection vary considerably in different regions of the world and reflect the progress of local epidemics, fueled by distinct modes of transmission, socioeconomic environments, and behavioral factors (168). Sub-Saharan Africa accounts for over 60% of the current cases of HIV infection and for 75% of the world's HIV-infected women and children, although it contains only 10% of the world's population. The overall prevalence of disease in adults between 15 and 49 years of age in sub-Saharan Africa is approximately 6%, but many countries in this region have prevalences exceeding 10%. Although the prevalence of infection has stabilized in some regions of Africa, this reflects approximately equal rates of incidence and mortality. Africa continues to account for the majority of new cases in the world. Asia accounts for over 20% of the world's cases of HIV-1 infection, with over 6 million cases as of the 2006. The majority of these have occurred in India. Latin America accounts for over 4% of the world's cases of HIV infection, with 1.6 million cases. Although the adult prevalence in Europe is less than 1%, regions in Eastern Europe have experienced marked recent increases attributable to high rates of injection-drug use. Eastern Europe and Central Asia now account for 1.6 million cases. North America accounts for approximately 4% of the world's cases of HIV infection.

Transmission

Transmission of HIV occurs through direct contact with infected body fluids, including blood and blood products (169), semen (169), vaginal and cervical secretions (170), amniotic fluid (171), and breast milk (172). Despite detection of HIV-1 RNA in saliva and tears (173, 174), there have been no documented cases of transmission via these body fluids. Transmission most commonly occurs during sexual contact with the exchange of semen, genital secretions, or blood from an infected individual to the uninfected partner. Unprotected receptive anal intercourse, with associated mucosal trauma, carries the highest risk of sexual transmission. In the majority of instances, heterosexual transmission occurs during vaginal intercourse, although cases have occurred after fellatio. Sexual transmission is facilitated by the presence of underlying sexually-transmitted diseases including chancroid, herpes genitalis, and syphilis, which disrupt the integrity of the skin or mucosal linings (175, 176).

Transmission is also more likely when the source partner is acutely infected and the concentration of virus in genital secretions is highest (177). The exact mechanism of sexual transmission is unclear. The form of the transmitted inoculum may be cell-associated virus or cell-free virions. How HIV breaches the mucosal barrier is also uncertain; the possibilities include facilitation at areas of disruption in the mucosa, as noted above, transcytosis of virions through epithelial cells with release into the lamina propria, or uptake of virions into Langerhans cells with transinfection of T cells as discussed above.

Infection also occurs through direct inoculation of infected blood, particularly re-use of contaminated needles, transfusion of infected blood products, and transplantation of infected tissues. The risk of HIV-1 transmission, following occupational percutaneous exposure to infected blood via a contaminated needle, is approximately 0.3% (178, 179). The likelihood of transmission is influenced by many factors, including the type of needle (hollow versus solid bore), the depth of penetration, the volume of the inoculum, the amount of infectious virus in the inoculum, and the post-exposure use of antiretrovirals.

The third primary mode of transmission of HIV-1 is from an infected mother to her child during pregnancy, delivery, or breastfeeding. The risk of maternal-fetal transmission is 13 to 40% in the absence of antiretroviral therapy (180), but this can be significantly reduced by the prevention approaches discussed below.

Patterns of Transmission

The primary modes of transmission vary in different regions and populations. The mode of transmission in sub-Saharan Africa is heterosexual in 90% of cases; almost 60% of the HIV-positive individuals in this region are women. This region also accounts for 87% of the world's total of HIV-infected children, in whom infection is acquired perinatally.

The pattern of spread of HIV in South and Southeast Asia is exemplified by the epidemic in Thailand, which began among injection-drug users and sex workers, then spread into the general population. Peak prevalences reached 10% among military recruits and 6.4% in prenatal clinics by 1993–1994, but they have declined subsequently due to control measures like condom use in commercial sex establishments (168). Heterosexual transmission plays a major role in the epidemics in Southeast Asia, as evidenced by the fact that 25% of the infected adults are women. In Latin America, the initial epidemic primarily involved men having sex with men. However, injection-drug use and heterosexual transmission have become significant modes of spread in this region.

The dramatic rise in incidence rates in Eastern Europe and Central Asia has been fueled by injection-drug use, primarily among men in the Ukraine and countries of the former Soviet Union (181). Eastern Europe has also been the site of nosocomial outbreaks of HIV infection caused by improper reuse of medical equipment. In Western and Northern Europe, the initial epidemic involved men having sex with men. More recently, the incidence of infection in other populations has increased, with heterosexual contact and injection-drug use accounting for an increasing number of cases.

In the United States, both the overall prevalence and incidence of new HIV infections remain greatest among homosexual and bisexual men, with disproportionate case numbers in African-Americans. The proportion of infections in women in the United States has progressively increased to a quarter of all cases, with higher rates in African American and Hispanic women. In contrast, the HIV infections diagnosed among infants declined dramatically from 907 cases in 1992 to 53 in 2011, attributable to the treatment of infected women, prenatal screening, and peripartum chemoprophylaxis.

PATHOGENESIS

Incubation Period

In classic terms, the incubation period is the interval between acquisition of the infection and onset of the illness.

For HIV-1 infection, the term is problematic in the sense that a symptomatic illness, called primary HIV-1 infection, develops in some infected individuals within one to four weeks after exposure, while clinical immunodeficiency (AIDS) typically appears after a prolonged asymptomatic period measured in years following primary infection. During this time, active viral replication and critical pathophysiologic changes occur. Patients with advanced HIV-1 infection show numerous immunologic abnormalities, the most prominent of which are severe quantitative and qualitative defects in the $CD4^+$ T lymphocyte compartment. Much of the decline in $CD4^+$ T-cell counts occurs during the asymptomatic period between initial infection and the development of clinically-apparent immunodeficiency. In adults, the average length of this asymptomatic period is 10 years. Opportunistic infections (OI) by organisms that do not cause disease in immunocompetent individuals herald the development of AIDS. These usually do not occur until the level of $CD4^+$ T cells has dropped from the normal levels of 600 to 1000 cells/μl to below 200 cells/μl. The degree of loss of $CD4^+$ T cells is an excellent predictor of progression to AIDS, and a $CD4^+$ T-cell count below 200 cells/μl is now considered to be an AIDS-defining condition. Susceptibility to particular opportunistic infections appears in a predictable way, such that some infections appear as the CD4 count falls below 200 cells/μl (*Pneumocystis jiroveci* pneumonia), while other infections are seen only in patients whose CD4 counts have fallen to below 100 cells/μl (*Mycobacterium avium* complex infection) or 50 cells/μl (cytomegalovirus [CMV] retinitis). These findings suggest that the loss of $CD4^+$ T cells is central to the development of clinical immunodeficiency. Therefore, understanding the mechanism of CD4 depletion is the central problem in AIDS pathogenesis.

Patterns of Virus Replication

Organ and Cell Specificity

In untreated individuals virus is detected in the blood throughout the course of HIV-1 infection, but virus replication occurs predominantly in the peripheral lymphoid organs, especially the spleen, lymph nodes, and gut-associated lymphoid tissue (182, 183). However, HIV-1 virions can infect $CD4^+$ cells in any tissue. Target cells include not only mature $CD4^+$ T cells in lymphoid tissues but potentially also developing T cells in the thymus and the ubiquitous tissue macrophages, which express low amounts of CD4. While fusion and entry occur if appropriate coreceptors are expressed, subsequent steps in the replication cycle depend upon the cell type ($CD4^+$ T cell versus macrophage) and the state of activation of the cell.

$CD4^+$ T Lymphocytes

The cellular dynamics of infection of $CD4^+$ T lymphocytes by HIV-1 are illustrated in Fig. 7. $CD4^+$ T cells are heterogeneous with respect to activation state (resting versus activated) and previous antigen exposure (naïve versus memory), and HIV-1 pathogenesis must be considered in the context of this heterogeneity. Normally, the majority of mature $CD4^+$ T lymphocytes are in a resting G_0 state. These resting T cells are among the most quiescent cells in the body and are simply waiting to encounter antigen. About half are memory cells, which have previously responded to some antigen, while the remainder are naïve T cells.

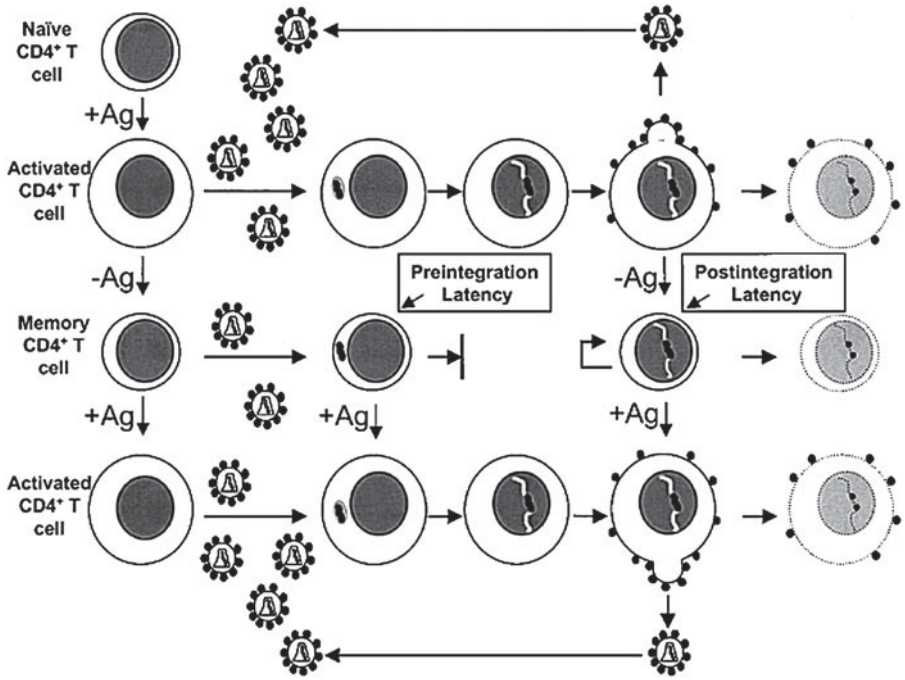

FIGURE 7 Cellular dynamics of HIV-1 infection of $CD4^+$ T cells. Successive steps in the life cycle of the virus are indicated by horizontal arrows. Transitions between resting (small) and activated (large) $CD4^+$ T cells are illustrated by vertical arrows. HIV-1 can infect resting and activated $CD4^+$ T cells, but integration of the reverse-transcribed HIV-1 provirus, which is necessary for virus production, occurs only in antigen-activated T cells. Productive infection requires antigen-driven activation of recently infected resting $CD4^+$ cells or infection of antigen-activated $CD4^+$ T cells. Productively-infected cells generally die within a few days from cytopathic effects of the infection, but some survive long enough to go back to a resting state, thereby establishing a stable latent reservoir.

The frequently-transmitted CCR5-tropic (R5) forms of HIV-1 can bind to, and fuse with, activated $CD4^+$ T cells and a small subset of resting memory $CD4^+$ T cells (184). CXCR4-tropic (X4) viruses can enter essentially all $CD4^+$ T cells due to the broad expression of CXCR4 in CD4+ T cells. Resting $CD4^+$ T cells do not generally support productive infection due to numerous blocks in the viral life cycle. SAMHD1, a host cell restriction factor, is a deoxynucleoside triphosphate (dNTP) hydrolase that restricts HIV-1 in nondividing cells by hydrolyzing dNTPs required for reverse transcription (77, 78, 185). Its action may account for the fact that reverse transcription occurs very slowly in resting $CD4^+$ T cells (186). As reverse transcription slowly proceeds in these cells, the resulting viral DNA can be sensed by a cytoplasmic DNA sensor, interferon-γ-inducible protein 16 (IFI16), which triggers an innate immune response, leading to caspase-1 activation and pyroptosis, an inflammatory form of cell death (187–189). Even if reverse transcription is completed in resting $CD4^+$ T cells, entry of the preintegration complex containing the reverse transcribed HIV-1 DNA into the nucleus may not occur (190). Thus, in resting T cells, reverse-transcribed HIV-1 genomes reside in the cytoplasm for a finite period of time (days to weeks) before the preintegration complex becomes nonfunctional. If the T cell is activated by antigen before the preintegration complex becomes nonfunctional, then the subsequent replicative steps can occur. In this sense, resting T cells carrying unintegrated HIV-1 DNA represent a latent reservoir for the virus known as preintegration latency (191). In untreated, asymptomatic HIV-1-infected individuals, most of the viral DNA present in resting $CD4^+$ T cells is in this unintegrated form (192, 193). Because transcription of unintegrated viral DNA in the cytoplasm cannot occur, latently-infected cells carrying this form of viral DNA presumably escape detection by immunologic mechanisms. In addition, there are blocks at subsequent steps in the virus life cycle. For example, lack of key host-cell transcription factors NFκB and NFAT in the nucleus of resting CD4+ T cells ensures that resting $CD4^+$ T cells probably do not become productively-infected without some form of activating stimulus.

Following encounter with antigen, resting $CD4^+$ T cells undergo blast transformation and enter a state in which they are highly susceptible to productive infection by HIV-1. In activated T cells, there is no block to nuclear import. Thus, the infection progresses rapidly to integration, viral gene expression, and virus production. Gene expression from the HIV-1 LTR is dependent upon host transcription factors, such as NFκB that are upregulated in activated T cells (194). This adaptation allows HIV-1 gene expression and virus production to be dramatically increased in activated $CD4^+$ T cells and largely shut off in resting $CD4^+$ T cells.

From this point, there are several possible fates for infected cells (Fig. 7). *In vitro* studies have shown HIV-1 infection can be highly cytopathic for activated $CD4^+$ T cells and can induce cell killing by mechanisms that are described below. Some of these cells can also be destroyed by immunologic mechanisms, including HIV-1-specific cytolytic T lymphocytes. The resulting half-life of productively-infected $CD4^+$ T cells is relatively short, generally 1.5 days. However, some of the productively-infected $CD4^+$ T cells escape both the viral cytopathic effects and immunologic effector mechanisms and revert to a resting memory state carrying integrated provirus (195). This is a reflection of the normal physiology of T-cell activation; a fraction of the T cells that respond to any given antigen survive and enter the long-lived pool of memory T cells. In a resting state, these memory $CD4^+$ T cells are likely to have little or no virus gene expression and therefore go unrecognized by the immune system. Because the viral DNA is in an integrated state in these cells, it is highly stable. Thus these cells provide a long-term, stable latent reservoir for the virus, a phenomenon termed postintegration latency (193, 195). The distribution of HIV-1 in various subsets of T cells reflects the physiologic factors described above (196). Levels of HIV-1 infection are lowest in naïve CD4+ T cells. Latent virus persists in resting memory CD4+ T cells. The virus persists in various subsets of memory cells including central memory cells, effector memory cells, and transitional memory cells (197). These subpopulations have somewhat different trafficking patterns and proliferative potential. Importantly, they all harbor latent HIV but to different extents in different patients.

As discussed below, the pool of latently-infected cells represents a major barrier to curing HIV-1 infection. Upon subsequent exposure to antigen, these cells will become activated and release infectious virus. Thus, antigen plays a critical role in driving $CD4^+$ T cells into states in which they are susceptible to productive infection by HIV-1 and subsequent destruction by viral cytopathic effects or immune mechanisms (Fig. 7).

Macrophages

The cellular dynamics of HIV-1 infection are different in macrophages as compared to $CD4^+$ T cells. HIV-1 can replicate in macrophages (198, 199), although integration and replication can occur only in dividing cells for most retroviruses. Specific amino acid sequences in the HIV-1 Gag, Vpr, and integrase proteins, and even a portion of the reverse-transcribed HIV-1 DNA, all have been reported to participate in targeting the viral preintegration complex for nuclear import, permitting integration and replication in nondividing cells, such as macrophages (59, 123, 200). Infection is not cytopathic in macrophages, and *in vitro* studies suggest that infected macrophages can continue to produce virus over long periods of time (198, 199). Infected macrophages may thus serve as a reservoir of virus *in vivo*; however, the role of macrophages as an obstacle to potential cure remains unresolved. The detection of viral DNA in macrophages from subjects appropriately treated with antiretroviral drugs may reflect phagocytosis of dead or dying infected CD4+ T cells that are infected (201). Virus production by macrophages is particularly apparent late in the course of disease when few $CD4^+$ T cells remain and in the setting of opportunistic infections (202).

Time Course of the Infection

The natural history of HIV-1 infection may be divided into three phases (Fig. 8). During the initial phase, known as primary or acute HIV-1 infection, virus replication produces a viremia that spreads the virus throughout the body. Viremia is eventually reduced by the emergence of CD8 T-cell responses. These events occur during the first several weeks following exposure to HIV-1. In the 50 to 70% of individuals who develop constitutional symptoms during primary infection, a transient illness resembling infectious mononucleosis appears two to three weeks after exposure (see below). During the acute illness, levels of genomic HIV-1 RNA in the plasma (a reflection of the free virion concentration) are usually greater than 10^6 copies/ml and can be as high as 10^8 copies/ml. Antibodies to HIV-1 are initially absent, and seroconversion usually occurs within a few weeks after onset of the acute illness.

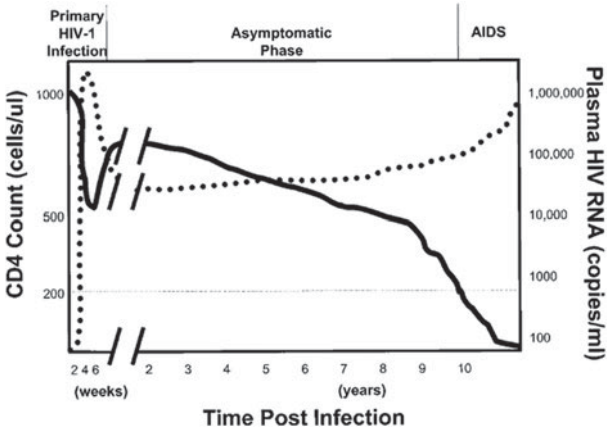

FIGURE 8 The three stages of disease in a hypothetical case of HIV-1 infection.

As the immune response to HIV-1 develops, there is a dramatic reduction in the level of viremia. Typically viremia falls to a lower plateau level (the "set point"). Set point values for plasma HIV-1 RNA are usually between 10^3 and 10^5 copies/ml. The $CD4^+$ T-cell count is typically reduced during symptomatic primary HIV-1 infection, reflecting both virus-induced depletion and sequestration of circulating $CD4^+$ T cells in lymphoid organs. After the acute illness resolves, peripheral $CD4^+$ T-cell counts generally rise again but usually not to preinfection levels.

During acute infection, there is rapid and dramatic depletion of $CD4^+$ T cells from the gut-associated lymphoid tissue (183, 203, 204). This early CD4 depletion in the gut is much more pronounced than CD4 depletion in other organs. The early loss of $CD4^+$ T cells from the gastrointestinal mucosa allows translocation of microbial products, which causes immune system activation and, in turn, contributes to CD4 depletion and end-organ disease (205).

Primary HIV-1 infection provides clues regarding the importance of various immune effector mechanisms in controlling HIV-1 infection. Virus-specific CTL appear early and likely represent a critical host factor in the control of acute HIV-1 infection (206, 207). In rhesus monkeys depleted of CD8+ T cells, the viremia of primary SIV infection is not brought under control, and the animals progress quickly to AIDS (208). Thus it is very likely that, through the lysis of infected cells and perhaps also through the release of chemokines like MIP-1α, MIP-1β, and RANTES that inhibit HIV-1 entry, CTL help to reduce the level of circulating virus to the lower levels that are characteristic of the asymptomatic phase of infection.

The initial seeding of the latent reservoir occurs during acute infection. In the SIV model of HIV-1 infection, a stable reservoir is established as early as day 3 postinfection, since prolonged administration of suppressive antiretroviral therapy beginning on day 3 postinfection is not curative and viral rebound occurs when treatment is stopped (209).

The second phase of HIV-1 infection is the long asymptomatic period between primary infection and the development of clinical immunodeficiency. The most important and characteristic pathophysiologic feature of the asymptomatic phase of HIV-1 infection is the gradual loss of $CD4^+$ T cells, although virus replicates continuously during this period. As primary infection resolves, plasma virus levels fall to a relatively stable steady-state level, which is different in different patients and which determines the rate of disease progression (210). The higher the set point of plasma HIV-1 RNA (often referred to as the viral load), the more rapidly the patient will lose $CD4^+$ T cells and progress to AIDS. The plasma HIV-1 RNA level determines how rapidly CD4 cells will be lost, and the CD4 count reflects the degree of impairment of immunologic function and the risk of opportunistic infections.

There is tremendous individual variation in the length of the asymptomatic period in different infected individuals. Some individuals progress to AIDS within two years of infection, whereas others termed long-term nonprogressors (LTNP) have lived with HIV-1 infection for over 20 years without experiencing significant CD4 depletion. Many individuals in this category eventually do progress to AIDS in the absence of antiretroviral treatment. Nevertheless, a subset of LTNP, termed "elite controllers," actually have no measurable viremia in the absence of treatment (211). Many of these individuals harbor forms of HIV-1 that appear to be replication competent, but it appears that the virus is held in check by immunologic mechanisms (212). These individuals frequently carry the class I MHC allele HLA B*5701, suggesting a role for cytolytic T lymphocytes (213). It is likely that both virologic and host immunologic factors (discussed below) play a role in limiting HIV-1 replication and disease progression in elite controllers and LTNP. Some LTNP are infected with strains of HIV-1 that are defective in accessory genes like *nef* (214). Polymorphisms in structural and regulatory regions of other chemokine receptors or chemokine genes have subtle but significant effects on the rate of disease progression (215, 216).

Viral loads are, on the average, lower in women in the first five years after infection, although rates of disease progression are similar to those observed in men (217). Rates of disease progression may be influenced by environmental factors, particularly concurrent infections. Although a direct relationship between rate of progression and concurrent infections has not yet been clearly established, the activation of $CD4^+$ T cells in response to other infections provides the virus with an increased number of target cells, allowing transient spikes in viremia, which can also be observed after immunizations with recall antigens (218).

Another important factor in disease progression is the evolution of viral variants with substitutions in the envelope protein leading to a change in the pattern of chemokine receptor utilization, specifically from CCR5 to CXCR4. These so-called X4 viruses have a potentially wider host-cell range due to the broader distribution of CXCR4 and have been associated in some studies with more rapid replication rates and higher cytopathic potential. The appearance of X4 viruses is temporarily associated with more rapid CD4 cell decline (219). The rate of disease progression is thus influenced by the characteristics of the infecting virus, host genetic factors influencing virus entry and antiviral immune responses, and possibly environmental factors related to immune system activation.

Factors in Disease Progression

Although rates of progression differ, the fundamental relationship between viral load and the rate of CD4 cell loss remains a central feature of AIDS pathogenesis, indicating that viral replication is the driving force for CD4 depletion. However, the exact mechanisms are still uncertain. For example, it is unclear whether the fraction of cells infected is high enough to account for the depletion of the entire $CD4^+$ T-cell compartment as a consequence of direct infection. At

any given time, only a small fraction of $CD4^+$ T cells are productively-infected. Thus, it is likely that additional mechanisms contribute to $CD4^+$ T-cell depletion. These include alterations lymphoid tissue microenvironments and chronic immune activation.

Histopathologic Changes

During the asymptomatic phase of the infection, there are important changes in the structure and function of the peripheral lymphoid organs (182). Early in the asymptomatic period, the lymph nodes show characteristics of immune activation. Scattered productively-infected $CD4^+$ T cells are seen throughout the lymph nodes. The B-cell areas of the lymph nodes show a pattern of follicular hyperplasia indicative of intense B-cell stimulation. Virus particles are readily detected in the germinal centers where they are found associated with the network of follicular dendritic cells (FDC). These cells express Fc receptors and three types of complement receptors (CR1, CR2, and CR3) and, as a result, are capable of binding antigens that have bound antibody or activated complement. FDC may serve as filters that trap virus particles and thereby lower the level of infectious virus in the circulation. FDC are not susceptible to HIV infection; however, they play an important role in the activation of B lymphocytes in response to antigen. During the asymptomatic phase of HIV-1 infection, there is progressive disruption of the normal architecture of the lymph nodes, with loss of the FDC network and follicular involution (182). Fibrotic changes in the lymph node microenvironment may impair the homeostatic mechanisms that maintain T-cell populations (220). The loss of FDC may, in part, be responsible for the abnormal B-cell function observed in HIV-1-infected individuals. In contrast to FDC, blood-derived dendritic cells (DC), which have an important role in the presentation of antigens to T cells, can bind virus and transmit the virus to the $CD4^+$ T cells with which they interact during the course of an immune response (221). Following stimulation with inflammatory cytokines, immature tissue DC migrate to the lymph nodes where they present antigens taken up in the tissues to T cells in the lymph nodes. DC may carry virions bound via dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) to the nodes where they mediate infection of $CD4^+$ T cells (154), but the importance of this pathway of infection remains unclear.

Viral Dynamics

Analysis of changes in the level of viral RNA following the initiation of antiretroviral therapy has provided a striking picture of the very dynamic nature of the infection (222) (Fig. 9). All current antiretroviral drugs have the effect of blocking the new rounds of infection of susceptible cells without inhibiting release of virions by cells that are already infected. Following the initiation of therapy, plasma virus levels drop by approximately two logs within less than two weeks, indicating that the half-life of plasma virus is very short (now estimated to be on the order of minutes) and that the half-life of most productively-infected cells is also very short (1.5 days) (51, 223). Thus activated $CD4^+$ T cells that are only recently infected produce most of the plasma virus (224). Continuous new rounds of viral replication sustain the infection.

Because the rates of clearance and decay are largely independent of stage of disease and other factors, the level of viral RNA in the plasma reflects the rate of virus production. Because the half-life of free virus particles is extremely short,

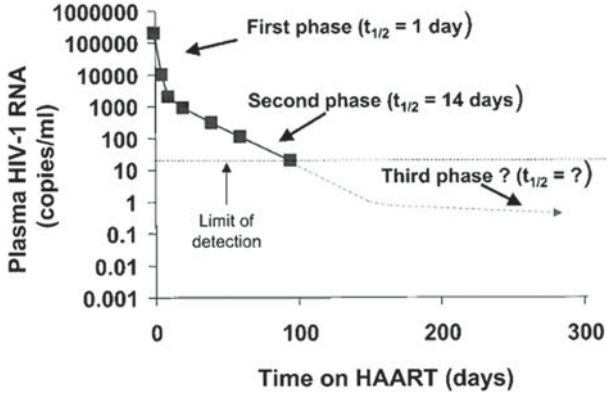

FIGURE 9 Hypothetical plot of plasma virus in a patient who is started on an effective regimen of drugs that block infection of new cells. Plasma virus titer drops rapidly in the first two weeks of treatment, reflecting the short plasma half-life of the virus and the short half-life of most of the productively-infected cells. These cells appear to be activated $CD4^+$ T cells. The decline in plasma virus shows a second, slower phase, which is due to turnover of cells infected before initiation of therapy. These may be persistently infected macrophages or $CD4^+$ T cells that are in a lower state of activation. Alternatively, this RNA could represent the clearance of virions that had accumulated in the germinal centers of lymphoid tissue. The second phase brings the viral load down to below the limit of detection, but the virus persists in reservoirs, including an extremely stable reservoir of latent virus in resting memory $CD4^+$ T cells. Reproduced from reference (484) with permission.

the steady-state level of viral RNA genomes in the blood reflects very recent virus production (106). The magnitude of the first phase of decay indicates that infected cells with longer half-lives, such as chronically infected macrophages, make a relatively minor contribution to plasma viremia in untreated individuals.

After the rapid initial phase of decay, there is a second slower phase of decay, reflecting either the clearance of virions that have accumulated in the germinal centers or the decay of a longer-lived population of virus producing cells (225), perhaps infected macrophages or infected $CD4^+$ T cells that are in a lower state of activation (224). The second phase of decay usually reduces plasma virus levels to below the limit of detection of current assays (50 copies or fewer of HIV-1 RNA/ml of plasma). The rapid drop in viremia initially raised hopes that eradication of the infection with antiretroviral therapy might be possible (225). Resting memory $CD4^+$ T cells in the postintegration state of latency (Fig. 7) persist; however, even in patients who have responded well to highly-active antiretroviral therapy (ART) and have had no detectable free virus in the blood for several years (226–228). These cells appear to represent the major barrier to curing the infection with antiretroviral drugs (229). The reservoir of resting memory $CD4^+$ T cells with integrated HIV-1 DNA is established early in primary infection (230) and shows minimal decay in infected adults (229) and children (231). Although early treatment usually leads to a smaller reservoir, recent studies indicate that the reservoir decay rate is nevertheless extremely slow (232).

Several hypotheses have been proposed to explain the extraordinary stability of the latent reservoir. The first is that the stability is simply a reflection of the fact that the virus has taken up residence in memory T cells, which, by nature, survive for prolonged periods of time. In this sense, the

mechanism of HIV-1 persistence resembles that of Epstein Barr virus (EBV), which establishes latent infection in memory B cells with viral persistence exploiting a fundamental feature of the immune system, the long-term survival of memory T cells. The stability of the memory T-cell response is due not only to the long half-life of individual memory T cells, but also to the process of homeostatic proliferation in which memory T cells occasionally enter the cell cycle and divide. The proliferation of latently-infected cells could contribute to the stability of the latent reservoir. Recent studies of HIV-1 integration sites have provided direct evidence for the proliferation of infected cells (233, 234). In some cases, the integration of HIV-1 into genes associated with cell proliferation may actually have driven the observed clonal expansions. What is not yet clear from these studies is whether the integrated proviruses are functional, as the overwhelming majority of proviruses in resting CD4+ T cells are defective due to large internal deletions or APOBEC3G-mediated hypermutation (235).

A distinct, but not mutually exclusive hypothesis, is that the reservoir is continually reseeded by a low level of viral replication that may continue even in patients whose plasma virus levels are below the limit of detection (236–240). Even in patients who have suppression of viremia to below the limit of detection of ultrasensitive clinical assays (50 copies of HIV-1 RNA/ml), free virus particles are continuously present (236). Recent studies suggest that a new steady-state level of viremia is reached in patients on HAART, with average values of approximately 1 copy/ml (241). While all patients on HAART are continuously viremic, direct analysis of this residual viremia does not provide evidence for viral evolution (242, 243), and it is possible that the residual viremia simply reflects release of virus from latently-infected cells that become activated or from other stable reservoirs and not ongoing replication (244). If this is the case, then the use of even more intensive drug regimens would not accelerate the intrinsic decay rate of the latent reservoir, and, in fact, no study of treatment intensification to date has shown a decrease in residual viremia (245).

T Cell Dynamics and Mechanisms of CD4 Depletion

In infected individuals, the rate of $CD4^+$ T-cell loss exceeds the rate at which $CD4^+$ T cells are produced by thymic differentiation and clonal expansion of peripheral $CD4^+$ T cells. $CD4^+$ T cells may be lost through a number of potential mechanisms, some operative for noninfected cells. In addition, the possibility that the production of $CD4^+$ T cells is decreased in HIV-1 infection needs consideration. HIV-1 infection accelerates the thymic involution that normally occurs with age. The decline in naive $CD4^+$ and $CD8^+$ T cells in the peripheral blood of infected individuals has been interpreted as indicating a defect in thymopoeisis (246), but it is difficult to measure directly the rate at which new T cells are produced in the thymus. To monitor ongoing thymic production of new T cells, studies have quantitated T-cell receptor excision circles (TRECs) produced as a by-product of the VDJ recombination reactions that occur in the thymus as new T cells are generated (247). These DNA circles are stable in cells after the gene rearrangements occur that produce functional T-cell receptors. Lower than normal TREC levels in peripheral blood $CD4^+$ and $CD8^+$ T cells have been observed in some HIV-1-infected adults, with partial reversal upon treatment. Analysis of TRECs generated by rearrangement of the α and β chains of the T-cell receptor has provided convincing evidence for a defect in thymocyte proliferation in HIV-1-infected individuals (248).

Accelerated $CD4^+$ T-cell loss is a critical factor in $CD4^+$ T-cell depletion. Under some experimental conditions, HIV-1 infection of susceptible cell types *in vitro* results in death of the infected cell. Syncytia (multinucleated giant cells) may form by the fusion of infected cells expressing Env protein and noninfected cells expressing CD4 (249). Inclusion of noninfected $CD4^+$ T cells in short-lived syncytia provides a potential mechanism for $CD4^+$ T-cell depletion. The extent to which syncytium formation contributes to $CD4^+$ T-cell depletion *in vivo* is unclear.

In addition, under some conditions, HIV-1-infected T cells appear to die from infection independent of any cell-cell fusion events (250, 251). Most potential mechanisms for HIV-1-induced single-cell killing involve the Env glycoprotein, which is poorly tolerated by many cell types. The fusogenic properties of the Env protein are an important determinant of the intrinsic toxicity of this protein for host cells (250). Other HIV-1 proteins, including Nef, Vif, and Vpr, have also been implicated in the death of infected cells (252, 253). Nonproductive or abortive infection of resting CD4+ T cells may lead to their rapid death through the process of pyroptosis (187–189). In these cells, the viral DNA intermediates, generated during reverse transcription, appear to be sensed by innate immune sensors, resulting in the triggering of cell death pathways.

Another obvious mechanism for the loss of $CD4^+$ T cells involves the destruction of such cells by components of the immune system, particularly $CD8^+$ CTL. As is discussed below, the natural immune response to HIV-1 infection includes a strong $CD8^+$ CTL response, and it is quite likely that $CD8^+$ CTL mediate the destruction of infected cells *in vivo*. The destruction of productively-infected cells by CTL is beneficial to the host because it leads to a more rapid cessation of virus production from cells that are destined to die. CTL provide a strong selective force that leads to the rapid evolution of viral variants with mutations in key epitopes recognized by CTL (see below). Nevertheless, while CTL are clearly important in controlling viral replication *in vivo*, it has been difficult to demonstrate that they actually shorten the lifespan of productively-infected cells (254).

During HIV-1 infection several interesting reactions can potentially cause the loss of $CD4^+$ T cells that are not infected. As discussed above, disruption of the architecture of the lymph nodes may contribute to CD4 depletion. Fibrosis is commonly observed in the lymph nodes of patients with advanced infection, and it is likely that the disruption of the microenvironment where CD4+ T cells reside contributed to CD4 depletion. HIV-1 infection is also associated with high levels of immune activation, and activated T cells are susceptible to apoptosis (255). *In situ* studies of lymph nodes from HIV-1-infected children and SIV-infected rhesus monkeys have shown that cells undergoing programmed cell death are distinct from cells productively-infected with virus, supporting the notion that indirect cell killing mechanisms may contribute to CD4 depletion (256). Rates of T-cell proliferation are increased several fold, and steady-state considerations suggest that this increased rate of $CD4^+$ T-cell proliferation must be more than balanced by an increased rate of destruction of T cells in the periphery (257, 258). The proximal cause of the immune hyperactivation seen in untreated HIV-1 infection remains unclear. One theory suggests that microbial translocation from the gastrointestinal tract contributes to the activation. Continuing

exposure to HIV and to other viruses, such as CMV are likely to also contribute to immune activation.

In addition to the depletion of $CD4^+$ T cells, qualitative defects in the function of the surviving $CD4^+$ T cells and on B-lymphocyte function. While normal absolute numbers of circulating B cells are found in HIV-1-infected individuals, circulating levels of immunoglobulins are high, reflecting polyclonal B-cell activation, but antibody responses to specific immunogens are very poor, particularly in patients with AIDS. Part of the B-cell defect may be intrinsic and not simply a consequence of defects in $CD4^+$ T cell help. However, the precise mechanisms remain obscure.

Immune Responses

Immunodeficiency develops in HIV-1 infected individuals despite the presence of readily detectable B and T lymphocyte responses to HIV-1. Virtually all infected individuals develop antibody responses to several of the protein products of the HIV-1 genome. Even more striking is the finding that most infected individuals also have very high levels of virus-specific CTL. On the other hand, $CD4^+$ T-cell responses are generally reduced. Current research is focused on understanding which elements of the immune response are the most important in controlling viral replication and why the response as a whole is not more effective in eliminating the virus.

Antibody Responses

All infected individuals develop readily measurable antibody response to HIV-1. Only antibodies to the extracellular portion of the Env glycoprotein can neutralize the virus. In general, levels of neutralizing antibodies are low even when high levels of anti-Env antibodies are present, indicating that many are not neutralizing. When neutralizing antibodies do arise, the virus can escape by accumulating mutations in the *env* gene. Of the viral proteins, the Env glycoprotein gp120 shows by far the most sequence variability. Neutralization-resistant variants have been selected *in vitro* in the presence of neutralizing antibody and arise readily when HIV-1 infection of human T cells is maintained in immunodeficient SCID mice (259). Antibodies present in patient sera can neutralize autologous virus isolates obtained at earlier time points, but generally cannot neutralize the contemporaneous isolate (28, 260), which results from the rapid and continuous evolution of the ectodomain of Env as a consequence of the selective pressure of the neutralizing antibody response (261). B-cell depletion studies in the SIV model have provided evidence that neutralizing antibodies contribute to the control of viral replication during chronic infection.

Antibodies to gp120 become detectable in the sera of HIV-infected individuals within two to three weeks after infection (27); however, these early antibodies are not neutralizing. They appear to be directed against disassembled envelope glycoproteins, and they recognize the interactive regions of gp120 and gp41, which, although immunogenic, are not exposed in the assembled, trimeric, glycoprotein complex (25). Subsequently, antibodies appear that are neutralizing but are usually restricted in activity to the infecting strain (28). Finally, antibodies appear that have more broadly neutralizing activity against a variety of isolates but in relatively low titers. A major recent advance has been the development of methods for cloning the antibody gene encoding these broadly neutralizing antibodies (bNAbs) (262, 263). Many of these bNAbs interfere with the binding interaction between gp120 and CD4. Such antibodies recognize discontinuous epitopes, the key residues of which are located within the binding pocket for CD4 on gp120 (the so-called CD4BS epitopes). Other broadly neutralizing antibodies recognize epitopes that are near conserved structures involved in coreceptor binding (the so-called CD4i epitopes), membrane proximal epitopes, or epitope composed largely of gp120 oligosaccharides. A common feature of these bNAbs is that they have unusual structures, likely the result of a long period of co-evolution of the virus and the host immune response. Whether it will be possible to induce these antibodies in a vaccine setting is not yet clear.

CTL Responses

HIV-1 specific $CD8^+$ CTL are readily detected in healthy seropositive individuals and are sometimes detected in patients with AIDS (264, 265). As discussed above, CTL appear early in response to acute HIV-1 infection and help to control the high-level viremia characteristic of this stage of infection by lysing productively-infected cells. CTL may also control viral replication through the release of chemokines (266). Vigorous HIV-1-specific CTL responses are observed in many long-term survivors of HIV-1 infection (267). The frequency of HIV-1-specific CTL appears to decline as disease progresses. In the SIV system, $CD8^+$ T cells help to control viremia in both acute and chronic infection (208, 268). MHC genotype influences the rate of disease progression (269), and particular class I-MHC alleles have been associated with slower disease progression, probably as a result of the capacity of the relevant alleles to present conserved epitopes in HIV-1 proteins to CTL. Certain MHC-class I alleles are overrepresented among patients who control viremia without antiretroviral drugs. The breadth of the CTL response is also important, and perhaps, as a consequence, heterogeneity at the class I loci is also associated with slower disease progression (270).

The HIV-1-specific CTL response can lead to the evolution of epitope escape variants, which accelerate AIDS pathogenesis (271, 272). Viral escape from CTL responses can involve mutations that diminish viral fitness, as evidenced by reversion of the mutations upon transmission of the virus to a new host with a different HLA genotype (273). While mutational escape provides one mechanism by which HIV-1 persists in the face of an ongoing CTL response, the functional capacity of HIV-1-specific CTLs is compromised in patients with progressive disease. HIV-1-specific CTLs express the inhibitory receptor PD-1, which is associated with clonal exhaustion (274–276). In contrast, the maintenance of "polyfunctional" CTLs has been associated with slower disease progression (277). Escape mutations may complicate HIV-1 eradication efforts because patients who start treatment in the chronic phase of the infection will have archived viruses with escape mutations in major CTL epitopes in the latent reservoir (278).

Helper T-Cell Responses

The one component of the immune response that is not readily demonstrable in most infected people is the helper T-cell response to HIV-1 proteins. HIV-1-specific $CD4^+$ T cells are inactivated early in the course of the infection. They can be readily detected only in those rare individuals in whom the disease does not progress (long-term nonprogressors). Treatment of infected individuals with antiretroviral therapy early in primary HIV-1 infection facilitates this HIV-1-specific immune response to develop.

Central Nervous System Disease

Neurological problems are common in HIV-1 infection (279). In addition to opportunistic infections and malignancies

affecting the central nervous system (CNS), there is a unique dementia syndrome, HIV-associated neurologic dysfunction dementia (HAND), that appears to result from direct effects of HIV-1 on the CNS. HAND appears late in the course of disease, roughly coincident with the development of clinical immunodeficiency, and is an AIDS-defining condition.

The pathogenesis of HAND is complex and poorly understood but involves interactions between various types of infected and uninfected cells in the CNS. HIV-1 probably gains access to the CNS from the blood stream, which requires a mechanism for crossing the blood-brain barrier. This may occur either by direct infection of capillary endothelial cells (280), or, more likely, by ingress of infected monocytes/macrophages (281). This "Trojan horse" mechanism resembles the mechanism by which other lentiviruses gain access to the CNS. Although CNS disease does not become apparent until late in the course of infection, entry of HIV-1 into the CNS may occur very early. Studies using an artificial blood-brain barrier demonstrated that upregulation of adhesion molecules and proinflammatory cytokines are critical for transendothelial migration. Heightened trafficking may occur with peripheral activation of monocytes in late-stage HIV-1 infection, which is generally when HAND occurs. Proinflammatory cytokines like TNF-α may also alter the permeability of the blood-brain barrier to free virus (282).

The principal cellular target cells for HIV-1 replication in the CNS are brain macrophages and microglial cells (283). Both cell types are derived from peripheral blood monocytes and are presumed to undergo gradual turnover. In some cases, syncytia composed of numerous infected macrophages and microglial cells can be observed in the vicinity of blood vessels in the CNS. No convincing evidence exists for HIV-1 DNA in neurons, endothelial cells, or oligodendrocytes (284, 285). Progress in the understanding of the extent of infection within the CNS has been hampered by the obvious difficulty in obtaining tissue and by uncertainties with regard to the relationship between level of virus in the cerebrospinal fluid (CSF) and levels of virus in the brain parenchyma.

The development of HAND is not universal in advanced AIDS, suggesting that there may be viral, as well as host, genetic determinants of heightened risk. Indeed, distinct strains of HIV-1 isolated from both peripheral blood and the nervous system of the same individual can have different biological characteristics and cellular tropisms. Brain isolates tend to be more macrophage-tropic with specifically conserved regions in a portion of the envelope, the V3 domain (286, 287). These observations suggest that certain strains of HIV-1, having an increased propensity to invade (neurotropism) and cause damage in the nervous system (neurovirulence), might lead to clinically significant CNS involvement (288, 289).

How viral infection of brain macrophages and microglial cells leads to CNS dysfunction is still unclear. The simplest hypothesis is that infected cells release soluble products that damage other cells in the CNS. The long list of potential mediators includes viral proteins like gp120, Tat, and Nef, as well as inflammatory mediators such as TNF-α, NO, and prostaglandins (283). Concern exists that the virus might persist in the CNS and produce disease even in treated patients who have no detectable plasma virus, in part because of the limited CNS penetration of certain protease inhibitors. Other important factors include the active efflux of antiretroviral drugs through transporters including p-glycoprotein (290). While case reports, describing patients who had undetectable or low plasma HIV RNA levels yet significantly higher CSF HIV RNA levels, alerted clinicians to this possibility, there have been relatively few clinical examples of "CNS escape." In fact, significant reductions in the incidence rates of HAND have been noted since 1996 (291). HAART regimes can actually improve neuropsychological performance and radiological abnormalities in those with HAND (292). Turnover of infected microglial cells over the course of weeks to months may result in replacement by uninfected monocytes from the blood. Thus, as is the case with HIV-1-induced immune deficiency, HAND appears to be driven by active viral replication.

CLINICAL MANIFESTATIONS

The cardinal manifestation of HIV-1 infection is the progressive loss of $CD4^+$ T lymphocytes. The resulting defect in cellular immunity leads to development of the opportunistic infections and malignancies that characterize AIDS. In addition, certain organ-specific syndromes may be caused directly by the virus itself. A comprehensive discussion of the myriad complications of HIV-1 infection is beyond the scope of this chapter, and the reader is referred to the many excellent textbooks of infectious diseases and AIDS medicine for more detailed discussions of specific syndromes and opportunistic pathogens.

Major Clinical Syndromes

Primary Infection

Symptomatic primary infection with HIV-1 occurs in approximately 30 to 70% of infected individuals (293). Symptoms begin around 14 days after exposure and peak virus titers occur a week later (294). The most frequent symptoms include fever, pharyngitis, headache, arthralgias, myalgias, and malaise (Table 2). A nonpruritic, maculopapular rash on the face and trunk is also commonly observed. Generalized lymphadenopathy is a frequent finding. Mucocutaneous ulceration occurs and helps to differentiate primary HIV-1 infection from other viral syndromes. Oral candidiasis occurs frequently, and candidal esophagitis is well-documented (293). Other gastrointestinal symptoms can include nausea, vomiting,

TABLE 2 Frequency of signs and symptoms in patients with acute HIV-1 infection

	Frequency (%)			
Sign/symptom	Kinloch-de Loes[a] (n=31)	Daar[b] (n=40)	Sydney[c] (n=74)	Schacker[d] (n=41)
Fever	87	88	80	∽95
Lethargy/fatigue	26	n/a	67	∽90
Myalgia	42	60	63	∽60
Rash	68	58	53	∽30
Headache	39	55	51	∽55
Sore throat/Pharyngitis	48	43	51	∽70
Lymphadenopathy	6	38	43	∽50
Arthralgia	29	28	26	n/a
Diarrhea	32	n/a	32	∽45
Night sweats	n/a	50	n/a	∽45
Oral ulcers	13	8	30	n/a

[a]From reference (479).
[b]From reference (350).
[c]Group 3 from reference (480).
[d]Estimated from Fig. 1 in reference (481).

and diarrhea. Aseptic meningoencephalitis is the most common neurological manifestation of primary HIV-1 infection. Acute peripheral neuropathy, myelopathy, and mononeuritis multiplex are less-frequently observed (293). In the majority of patients, symptoms resolve within a month. Persistence of symptoms beyond 8 to 12 weeks, along with a severely depressed $CD4^+$ lymphocyte count, may be clues to unusually rapid progression of HIV-1 disease.

The virologic and immunologic aspects of primary HIV-1 infection are discussed in the Pathogenesis section. The principal laboratory abnormality is a decrease in the absolute $CD4^+$ lymphocyte count. In most patients there is also an increase in reactive CD8+ T lymphocytes. Hematologic abnormalities are uncommon except for mild thrombocytosis. Serum aspartate transaminase and alkaline phosphatase levels may be mildly elevated, but clinical hepatitis is an infrequent finding (293).

Progression to AIDS

In most patients, a prolonged asymptomatic period follows the resolution of primary infection. In the absence of antiretroviral therapy, the median time from acquisition of HIV-1 infection to AIDS is approximately 8 to 10 years. Use of the term "clinical latency" to describe this interval is misleading, given the presence of continuous virus replication and $CD4^+$ lymphocyte depletion. On average, there is a loss of 30 to 60 $CD4^+$ cells/μl per year, although in many patients $CD4^+$ lymphocyte counts may remain stable for several years followed by a period of rapid decline. Progression to AIDS within one to two years of primary HIV-1 infection occurs in less than 5% of patients. Rapid progression often follows severe primary infection and may be associated with transmission of syncytium-inducing (CXCR4 utilizing) variants of HIV-1 (295).

In many patients, fatigue and lymphadenopathy continue to be noted during the otherwise asymptomatic phase of HIV-1 infection. The occurrence of minor clinical events, such as oral hairy leukoplakia (secondary to EBV), oral or vaginal candidiasis, herpes zoster, and a variety of other dermatologic disorders, may be early signs of progression. Worsening fatigue, night sweats, and weight loss become more common with advancing disease. The risk of serious, potentially life-threatening opportunistic infections increases significantly at $CD4^+$ cell counts below 200/μl. For this reason, a CD4 count of less than 200/μl was selected as a diagnostic criterion of AIDS according to the 1993 revised CDC case definition (296).

Systemic and Organ-Specific Manifestations

Infection with HIV produces a variety of clinical manifestations affecting nearly every organ system. Improvement in most of these manifestations, following the institution of potent antiretroviral therapy, strongly implicates HIV replication or immune activation in their pathogenesis. Earlier initiation of antiretroviral therapy, as a result of changing treatment guidelines, has made these manifestations significantly less common in economically-advantaged countries, although examples are still seen among persons presenting with advanced disease and in resource-limited settings.

Dermatologic disorders, including pruritus and xerosis, are frequent symptoms in patients with early infection; a variety of noninfectious inflammatory conditions are commonly observed including seborrheic dermatitis, papular pruritic eruption, and eosinophilic folliculitis (297).

Neurologic manifestations of HIV infection include disorders of the central and peripheral nervous system, opportunistic infections, malignancies, vascular complications, and myopathies. The most frequently recognized "primary" neurologic manifestation of HIV infection is HAND, or the AIDS dementia complex, which occurs in up to 27% of patients with late-stage HIV disease (298). This syndrome involves progressive cognitive, motor, and behavioral deficits that usually begin in patients with moderately advanced HIV disease ($CD4^+$ cell counts below 200/μl). Computed tomography or magnetic resonance imaging studies of the brain usually show evidence for cortical atrophy, ventricular enlargement, and diffuse white matter abnormalities. Rapid clinical improvement in response to treatment argues for a central role of HIV in the pathogenesis of AIDS dementia (299).

Disorders of the peripheral nervous system can be classified as distal symmetric polyneuropathy, toxic neuropathy, inflammatory demyelinating polyneuropathy, progressive polyradiculopathy, and mononeuropathy multiplex (298). Up to 35% of patients with HIV infection develop signs or symptoms of distal symmetric polyneuropathy in later stages of disease (300). Symptoms begin as burning pain and numbness in the feet and may progress to the point that ambulation becomes impossible. Subjective complaints of distal polyneuropathy may precede objective findings on neurologic examination by several months. In more advanced cases there is diminished vibratory sensation and loss of ankle reflexes. Inflammatory demyelinating polyneuropathy is characterized by progressive weakness and areflexia resembling that of Guillain-Barré syndrome. This form of neuropathy, along with mononeuropathy multiplex, may occur during primary HIV-1 infection and is thought to have an autoimmune basis (298).

Nonspecific interstitial pneumonitis and lymphocytic interstitial pneumonitis (LIP) have been associated with HIV infection, as has primary pulmonary hypertension. The occurrence of LIP is most frequent in infants with mother-to-child transmission of HIV (see below). Clinical signs and symptoms of interstitial lung disease are similar to those in HIV-uninfected patients. Histological features include lymphocytic or mononuclear cell infiltration and the absence of known pulmonary pathogens (301).

Oral pathology attributable to HIV infection includes xerostomia, recurrent aphthous stomatitis, and gingivitis. Giant aphthous ulcers of the esophagus also occur but less frequently than aphthous stomatitis. Diarrheal illness is common in patients with advanced HIV disease and usually can be attributed to infection with specific enteric pathogens, such as cryptosporidium, microsporidia, or *M. avium* complex. Extensive HIV-1 infection occurs in gut-associated lymphoid tissue, suggesting a direct contribution of HIV-1 to intestinal dysfunction (302).

Endocrine dysfunction and metabolic disorders are well-described as complications of advanced HIV infection, including hypogonadism, loss of libido, and testicular atrophy. It is uncertain whether the primary abnormality is at the level of the hypothalamus or the testis. Fertility is decreased and the rate of fetal loss increased among women with advanced stages of disease (303).

Wasting syndrome in HIV infection is defined as the unintentional loss of more than 10% of body weight. When accompanied by constitutional symptoms for longer than 30 days, wasting, in the absence of opportunistic infection, or malignancy is sufficient to make a diagnosis of AIDS (296). In the developing world, the high prevalence of HIV-associated wasting has given AIDS the appellation "slim disease." In developing countries, some degree of HIV-associated wasting

occurs in nearly a third of otherwise asymptomatic patients (in the absence of effective antiretroviral therapy) and in 60 to 90% of patients with AIDS (319). As in starvation and cancer, death from wasting in AIDS occurs at 66% of ideal body weight (304).

Anemia and neutropenia are the most frequent hematologic disorders in HIV-infected individuals. Although a large number of drugs used in the treatment of HIV infection and its complications can cause bone marrow suppression, HIV infection per se is clearly associated with anemia and neutropenia, particularly in late-stage disease. In untreated patients with AIDS, anemia has been noted in 4% and neutropenia in 11% (305). The anemia is normochromic and normocytic, and iron studies are suggestive of the anemia of chronic disease. Although the hypergammaglobulinemia associated with HIV infection may lead to a positive Coombs's test in 20% of patients, other evidence in support of hemolysis as a cause of anemia in HIV infection is rare (306). Similarly, antigranulocyte antibodies detected in many HIV-infected patients do not appear to play a major role in the development of HIV-associated neutropenia. Progenitor cells for myeloid, erythroid, and megakaryocyte lineages from HIV-infected individuals have a reduced capacity for growth *in vitro*. The mechanisms by which HIV reduces the proliferative capacity of these progenitor cells are poorly understood.

In contrast to anemia and neutropenia, thrombocytopenia in HIV infection is usually due to immune-mediated destruction of platelets. Platelet-associated antibodies are detected in the majority of patients with HIV-associated thrombocytopenia, and examination of the bone marrow reveals increased numbers of megakaryocytes, suggesting peripheral destruction of platelets. The incidence and severity of immune thrombocytopenia appears to increase with diminishing $CD4^+$ cell count. Some degree of thrombocytopenia is present in 5 to 10% of patients at earlier stages of HIV disease and in up to 30% of patients with AIDS (306).

A variety of rheumatologic syndromes have been described in HIV-infected individuals, including Reiter's syndrome, psoriatic arthritis, polymyositis, vasculitis, and sicca syndrome (307). Inappropriate immune activation and polyclonal B-cell activation leading to hypergammaglobulinemia and autoantibody production are implicated in the pathogenesis of these HIV-associated syndromes, but precise pathogenetic mechanisms have not been defined.

HIV infection is also associated with a rapidly progressive form of glomerulosclerosis (HIV-associated nephropathy) leading to nephrotic-range proteinuria and renal insufficiency. The incidence of HIV-associated nephropathy is highest among African Americans and injection-drug users. Renal biopsy reveals collapsing focal segmental sclerosis of involved glomeruli, as well as tubulointerstitial changes accompanied by an interstitial mononuclear cell infiltrate and the presence of HIV-1 RNA in tubular epithelial cells and glomerular podocytes (308). Effective antiretroviral therapy has been associated with resolution of histologic changes. Despite reductions in HIV-1 levels in the kidney, persistence of viral RNA transcripts suggest that the kidney may serve as a reservoir for HIV-1 infection.

Complications

1. Opportunistic infections. The incidence and severity of opportunistic infections increases as cellular immunity wanes during the course of HIV-1 infection. The widespread use of potent antiretroviral therapy and chemoprophylaxis has markedly reduced the incidence of opportunistic infections in HIV-infected patients in the developed world. However, because of limited access or avoidance of care, patients continue to present, late in the course of HIV disease, with active opportunistic infections.

The $CD4^+$ cell count is the most useful marker for predicting the immediate risk of developing a particular opportunistic infection. Such complications are rare in patients with CD4 counts above 500 cells/μl. As the CD4 count drops below 500 cells/μl patients may begin to experience oral candidiasis, pneumococcal infections, and a host of cutaneous disorders including recurrent reactivation of herpes simplex virus, varicella zoster, dermatophyte infections, pityariasis, and onychomycosis. The risk of more serious opportunistic infections, such as *Pneumocystis* pneumonia, *Candida* esophagitis, reactivation of latent histoplasmosis and other systemic fungal infections, toxoplasma encephalitis, and cryptococcal meningitis, increases significantly as the CD4 count falls below 200 cells/μl. At $CD4^+$ cell counts under 50/μl, patients are at increased risk for the occurrence of disseminated infection with *Mycobacterium avium* complex, reactivation of cytomegalovirus infection, cryptosporidiosis, and progressive multifocal leukoencephalopathy (PML) due to John Cunningham (JC) virus infection.

Infection with HIV-1 significantly increases the risk of pulmonary and extrapulmonary tuberculosis. The HIV-1 pandemic has contributed to a 5- to 10-fold increase in the incidence of tuberculosis in resource-poor countries, where more than 80% of patients newly-diagnosed with tuberculosis are co-infected with HIV (309). In contrast to other opportunistic infections, the risk of tuberculosis is increased even in patients with well-preserved CD4 cell counts. A study in South African gold miners found that the incidence of tuberculosis doubled within the first year of HIV infection (310). Numerous other opportunistic infections have been catalogued in patients with advanced HIV disease, but are beyond the scope of this chapter.

2. Oncologic complications. The oncologic manifestations of HIV infection arise as opportunistic malignancies in the setting of severe immune deficiency. NonHodgkin's lymphoma (often associated with high-level EBV replication), Kaposi's sarcoma (associated with human herpesvirus 8 infection) (311), and cervical cancer (due to oncogenic serotypes of human papillomavirus [HPV] infection) are recognized as AIDS-defining cancers when they occur in HIV-infected patients. Anal carcinoma, although not formally considered an AIDS-defining cancer, is also caused by HPV infection. Other malignancies, including Hodgkin's disease, liver cancer, and stomach cancer also occur more often among HIV-infected patients than in the general population (312). By contrast, rates of common epithelial malignancies, such as breast, prostate, and colorectal cancer, are not increased.

3. Cardiovascular complications. HIV-1-infected patients have increased risk of myocardial infarction, compared to control populations matched for traditional cardiovascular risk factors (313–316). More striking is the finding that interrupting antiretroviral therapy significantly increases the risk of cardiovascular events, independent of CD4 cell count (317, 318). This increased risk is correlated with an increase in plasma levels of soluble markers of inflammation and coagulation like interleukin-6 (IL-6), high-specificity C-reactive protein (hsCRP), and D-dimer (319–321). In addition, adverse changes in surrogate markers associated with cardiovascular risk, such as flow-mediated vasodilatation, carotid intimal-media thickness, and coronary artery

calcification, occur in patients with HIV-1 infection (26, 27). The extent to which these changes can be prevented or reversed by antiretroviral therapy, anti-inflammatory drugs, and/or statins is an active area of current research.

HIV-1 Infection in Children

In the absence of preventive antiretroviral therapy, 15 to 30% of children born to HIV-infected mothers acquire HIV infection through mother-to-child transmission (322, 323). The course of HIV-1 infection is accelerated in children with vertically-acquired HIV-1 compared to HIV-infected adults. Because of the lymphocytosis of infancy, the absolute $CD4^+$ lymphocyte count may not accurately reflect functional immune status and must be adjusted for age. Without potent antiretroviral therapy, approximately 20% of infected infants develop AIDS in the first year of life, and 28% die before five years of age (324). In the developed world, the five-year survival rate for children who develop signs of HIV infection within the first five months of life is 45%, as compared to 74% for children with a later onset of disease (325). In lower socioeconomic countries, two-year mortality of HIV-infected children exceeds 50% (326). Lymphadenopathy, splenomegaly, and hepatomegaly are the most common signs of HIV infection in the first year of life. Growth failure and developmental delay are manifestations of HIV infection in children. Progressive encephalopathy is found in approximately 15% of infected children (327). Another unique manifestation is lymphoid interstitial pneumonitis, which occurs in 30 to 40% of children with HIV infection (327). Abnormalities in immunoglobulin synthesis predispose to recurrent pyogenic infections with organisms that are common causes of infection in young children, such as *S. pneumoniae*, *H. influenzae*, *Staphylococcus aureus*, and *Salmonella* sp. The most frequently-encountered opportunistic infections in children are Pneumocystis pneumonia, *Candida* esophagitis, disseminated CMV infection, cryptosporidiosis, and disseminated MAC infection (327).

Clinical Diagnosis

Before the discovery of HIV-1, AIDS was defined, in part, by the occurrence of opportunistic infections suggestive of immunodeficiency in patients not receiving immunosuppressive therapy and without a history of congenital immunodeficiency. This definition remains useful in helping to identify patients with advanced HIV-1 disease. Thus, the occurrence of opportunistic infections, such as *Pneumocystis* pneumonia, *Candida* esophagitis, cryptococcal meningitis, toxoplasmosis, or chronic ulcerative herpes simplex, in the absence of a known cause of immunodeficiency, should raise the possibility of HIV-1 infection. Recurrent or disseminated zoster, pneumococcal infection in a young adult, oral or recurrent vulgovaginal candidiasis, disseminated papillomavirus infection, persistent fever, night sweats, lymphadenopathy, weight loss, and chronic diarrhea all may be evidence of infection with HIV-1. Similarly, a diagnosis of HIV-1 infection should be entertained in patients with unexplained lymphopenia, anemia, or neutropenia, and in cases of idiopathic thrombocytopenia. In such cases, information should be sought from the patient to determine if he or she is at risk for HIV infection (e.g., men who have sex with men, current or prior injection-drug use, female sexual partners of bisexual men or injection-drug users, a history of unprotected sex with a new or unknown partner, or a history of transfusion or occupational exposure).

It is a difficult challenge to identify individuals during the asymptomatic stages of infection. Early diagnosis is essential in order to provide appropriate counseling and advice regarding modification of behaviors that may spread the virus to other individuals and to institute antiretroviral therapy prior to immune depletion. For this reason, the CDC recommends that HIV testing be performed as part of routine medical care at least once in all persons aged 13 to 64 years and annually in those at high risk of HIV infection (328). Presence of another sexually transmitted disease, infection with hepatitis B or C viruses, or active tuberculosis should prompt testing for HIV-1 infection.

The clinical diagnosis of primary HIV infection is particularly challenging due to the relatively nonspecific nature of the presenting signs and symptoms (Table 2), which may be confused with other viral infections including adenovirus or enterovirus infection, influenza, and CMV or EBV mononucleosis. This difficulty is further confounded by the observation that patients with acute HIV infection who seek medical attention usually do so in a primary care or emergency room setting, where clinicians may be less attuned to the possibility of primary HIV infection. In one study, approximately 1% of heterophile-negative sera from patients tested to exclude acute mononucleosis were found to be have high titers of HIV-1 RNA but negative results on HIV antibody testing, suggesting acute infection (329).

LABORATORY DIAGNOSIS

A diagnosis of infection with HIV can be made by virologic, serologic, or nucleic acid tests. For the majority of patients with clinical symptoms suggestive of HIV infection, and for those at high risk for HIV infection, diagnosis is straightforward. The broad application of HIV diagnostic tests to persons at little or no risk of acquiring HIV infection, however, requires an understanding of the performance characteristics of these assays.

Virus Isolation

HIV-1 can be cultured from plasma or peripheral-blood mononuclear cells (PBMC) of infected individuals. A positive culture provides direct evidence of HIV-1 infection, but virus culture is rarely necessary to establish a diagnosis. The overall sensitivity of PBMC culture is 95% or more in patients with $CD4^+$ cell counts below 500/μl, but sensitivity is lower in patients with higher CD4 counts. Virus isolation is limited to research purposes.

Antigen Detection Assays

Circulating HIV-1 capsid (p24) antigen becomes detectable by immunoassay approximately 15 to 20 days after infection (Fig. 10). Detection of p24 antigen has been incorporated into HIV-1/2 antigen/antibody combination immunoassays to enhance detection of HIV-1 infection during acute infection, prior to the development of HIV-specific antibodies (see below). After seroconversion, p24 antigen is complexed with p24 antibodies and becomes undetectable in the majority of infected individuals. For this reason p24 antigen assays in isolation are not useful diagnostic tests in asymptomatic individuals at risk for HIV-1 infection.

Nucleic Acid Detection

Qualitative Assays for Proviral HIV-1 DNA

Evidence for HIV-1 infection can be established by demonstrating the presence of proviral DNA in PBMC. Assays for detecting proviral DNA employ the polymerase chain reaction (PCR) to amplify conserved sequences in the HIV-1 *gag* or *pol* gene, coupled to a detection step based on

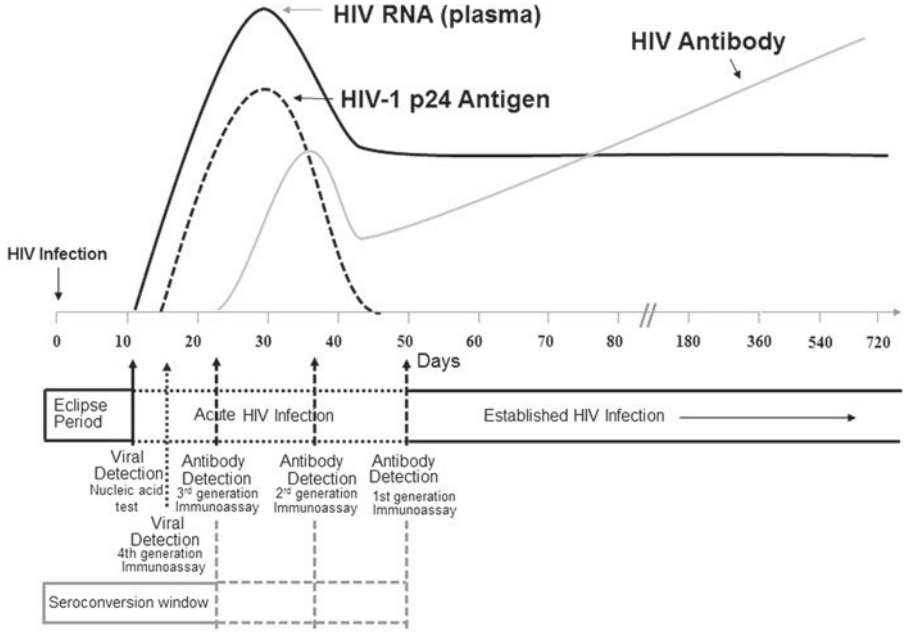

FIGURE 10 Order of appearance of laboratory markers of HIV-1 infection (228).

hybridization of a labeled oligonucleotide probe specific for the amplified gene sequences. With carefully standardized procedures and rigorous quality assurance and quality control, experienced laboratories can achieve 100% sensitivity and specificity.

Despite the excellent performance of these assays in proficiency panels, the sensitivity of HIV-1 DNA PCR assays in clinical practice is only 96 to 99%. As with virus culture and p24 antigen detection, sensitivity is lower in individuals with higher $CD4^+$ cell counts due to the lower titer of circulating infected PBMC. The greatest potential clinical utility of HIV-1 DNA PCR assays is in the early diagnosis of HIV-1 infection in neonates (see below). Clinical applications of these tests are relatively limited in adults, but occasionally DNA PCR testing may be helpful in persons with positive immunoassays but negative plasma HIV-1 RNA tests, as in elite controllers.

Assays for Quantifying Plasma HIV-1 RNA

Quantitative assay of HIV-1 RNA levels in plasma is used to monitor the course of disease and the response to antiretroviral therapy in patients already known to be HIV-1-infected. Several assays based on different methodologies have been approved by the FDA for clinical use.

Despite methodological differences, results of the commercially-available quantitative HIV-1 RNA assays are highly correlated (330, 331). The assays have a lower limit of quantification of 20 to 75 copies/ml, depending on the assay. The assay range can be extended by using larger volumes of plasma and pelleting virion particles prior to RNA extraction, but the precision with which plasma HIV-1 RNA can be quantified diminishes substantially at titers below 200 copies/ml. Serial testing of clinically stable patients not on antiretroviral therapy (or on a stable failing regimen) has shown the relative stability of plasma HIV-1 RNA levels over the short term (weeks to months), with a biological variation of approximately 0.3 to 0.4 $\log_{10}$ copies/ml (332). Thus, changes of greater than 0.5 to 0.7 $\log_{10}$ (3- to 5- fold) are likely to reflect significant changes in HIV-1 replication. Whereas earlier versions of these assays were specific for HIV-1 belonging to Group M, newer real-time PCR assays (COBAS AmpliPrep/COBAS TaqMan version 2, Roche Diagnostics; RealTime HIV-1, Abbott Molecular; Aptima HIV-1 Quant, Hologic) can also quantify HIV-1 Groups N, O, and P; they do not detect HIV-2 (333–335).

Clinical Utility of Plasma HIV-1 RNA Monitoring

Numerous studies have demonstrated the correlation of plasma HIV-1 RNA levels with stage of disease. Patients with AIDS or symptomatic HIV infection have significantly higher titers of plasma HIV-1 RNA than do those with asymptomatic infection, although HIV-1 RNA level and $CD4^+$ cell count are weakly correlated. Individuals with plasma HIV-1 RNA levels greater than 100,000 copies/ml within six months of seroconversion are 10 times more likely to progress to AIDS within five years than patients with lower levels of plasma HIV-1 RNA (336). Although plasma HIV-1 RNA levels are strong predictors of the risk of disease progression, they are weak predictors of the rate of CD4 count decline (Table 3) (337). This seeming paradox may be explained by the large variance observed in CD4 slopes and

TABLE 3 Association of plasma HIV-1 RNA level with decline in $CD4^+$ cell count and risk of AIDS and death

Plasma HIV-1RNA level (bDNA assay)	Change in $CD4^+$ cell count per year (cells/μl)	Progression to AIDS within six years	Death from AIDS within six years
< 500 copies/ml	−36.3	5.4%	0.9%
501-3,000 copies/ml	−44/8	16.6%	6.3%
3,001-10,000 copies/ml	−55.2	31.7%	18.1%
10,001-30,000 copies/ml	−64.8	55.2%	34.9%
> 30,000 copies/ml	−76.5	80.0%	69.5%

Adapted from reference (210).

the weak association between CD4 slope and risk of disease progression (338).

In the absence of treatment, plasma HIV-1 RNA levels may provide prognostic information in late stages of disease (339) and in children with perinatally-acquired HIV-1 infection (340). However, the CD4 count may be a better predictor of disease progression than is plasma HIV-1 RNA in patients with very low CD4 counts (below 50 cells/μl) (341).

The dynamic response of plasma HIV-1 RNA to treatment makes it possible to assess the effectiveness of antiviral therapy within a matter of weeks. A decrease in plasma HIV-1 RNA confers a significant reduction in risk of disease progression, independent of baseline plasma HIV-1 RNA level and CD4 count, and independent of the increase in CD4 count due to treatment (342). A $0.3\log_{10}$ (2-fold) reduction in plasma HIV-1 RNA levels confers a 30% reduction in the risk of progression to AIDS or death (343); a $1\log_{10}$ (10-fold) reduction reduces the risk of disease progression by approximately two-thirds (344). Although initial studies suggested that HIV-1 RNA was a more significant predictor of response to antiretroviral therapy than the change in CD4 count, subsequent studies make clear the prognostic importance of improvement in both markers (345, 346).

Sample Collection

Blood for plasma HIV-1 RNA testing should be collected into tubes containing EDTA as an anticoagulant and the plasma separated and stored frozen at −70° C until testing. Studies show that HIV-1 RNA is stable for up to 48 hours at room temperature in the presence of EDTA, but samples ideally should be processed within six hours after collection. Events leading to immune activation, such as vaccination or acute infectious illness, can transiently raise the plasma HIV-1 RNA level (218). Therefore, plasma HIV-1 RNA testing should not be performed within four weeks of an intercurrent infection or immunization. Because of differences between assay formats and commercial laboratories, the same laboratory should be used for serial tests on an individual patient.

Current treatment guidelines recommend obtaining plasma HIV-1 RNA level as part of initial patient evaluation. Virus load testing also should be performed immediately before and within two to eight weeks after initiating treatment to assess the initial response to a regimen. A decline in plasma HIV-1 RNA level of approximately 2.0 $\log_{10}$ is expected for treatment-naïve patients within eight weeks of starting an initial antiretroviral regimen, and plasma virus titers should fall to undetectable levels (below 50 copies/ml) by 16 weeks. However, more than 24 weeks may be required for plasma virus titers to fall below the limit of detection in patients with high levels of viremia (above 100,000 copies/ml). Declines of $1\log_{10}$ or more within eight weeks should be expected following a change in regimen due to treatment failure. Subsequently, plasma HIV-1 RNA levels should be repeated every three to four months to monitor the success of antiretroviral therapy. The frequency of testing can be reduced to once every six months in adherent patients with consistently-suppressed plasma viremia (347).

Plasma HIV-1 RNA Assays for Diagnosing Primary HIV-1 Infection

The earliest laboratory marker of HIV-1 infection is plasma HIV-1 RNA, which becomes detectable in plasma approximately 10 days after infection (348, 349). Quantitative plasma HIV-1 RNA assays are frequently used for diagnosing primary HIV-1 infection, although they are not approved for this purpose. These assays are highly sensitive (100%), but occasional false positive tests result in a specificity of only 97.4% (350). However, HIV-1 RNA levels are less than 3,000 copies/ml in nearly all false-positive assays, whereas HIV-1 RNA levels exceed 10,000 copies/ml in the great majority of patients with primary HIV-1 infection (351). Thus, plasma HIV-1 RNA testing should be considered in cases in which a history of recent exposure and symptoms consistent with acute HIV-1 infection provide a high index of suspicion. Qualitative HIV-1 RNA assays have been approved for HIV-1 diagnosis (Aptima HIV-1 RNA Qualitative Assay, Hologic Gen-Probe; Procleix Ultrio, Novartis Diagnostics) and are recommended for use in this setting (352).

Nucleic acid amplification tests also are used to screen donated blood to exclude presence of HIV-1 (and hepatitis B and C virus) infection. To maximize efficiency, samples from many donors are pooled and individual samples tested only if the pool tests positive (352). Four out of 12.6 million donations, that were p24-antigen-negative and seronegative, were found to be positive by nucleic acid amplification testing (1/3,150,000) (353).

Immunoassays

In most cases, infection is diagnosed by demonstrating the presence of antibodies specific for HIV-1 or HIV-2. Assays have been developed for detection of HIV antibodies in serum, whole blood, saliva, urine, and dried blood collected on filter paper. The appearance of IgM antibodies to HIV can be detected by 3rd and 4th generation HIV immunoassays approximately 25 days after infection (354, 355). The older 1st and 2nd generation assays, which detect only IgG antibodies, become reactive one to four weeks later (352). The time to serologic detection of HIV after initial infection can be reduced by seven days by the use of "4th generation" diagnostic tests, which combine detection of HIV antibodies and core (p24) antigen (Architect HIV Ab/Ab Combo, Abbott Laboratories; GS HIV Combo Ag/Ab EIA, Bio-Rad Laboratories) (356, 357). These assays have a sensitivity of 99.8 to 100% for HIV-1 and 100% for HIV-2 (358–360).

Serologic diagnosis is a two-stage process. Previously, sera that gave a positive reaction by an initial antibody screening assay (EIA, chemiluminometric, or rapid immunoassay) were retested to exclude the possibility of clerical or laboratory error, and repeatedly reactive sera were then tested by a confirmatory assay, such as a Western blot or immunofluorescence assay, to verify that reactive antibodies were directed against HIV antigens. Newer guidelines based on the availability of combination HIV-1/2 antibody/antigen detection assays have streamlined testing algorithms (Fig. 11). According to current guidelines, initial testing should be performed with an antigen/antibody combination immunoassay capable of detecting antibodies to HIV-1 and HIV-2, as well as HIV-1 p24 antigen (352). Such assays will detect infection in persons with established HIV-1 or HIV-2 infection, as well as with acute HIV-1 infection. Samples that test positive in this initial step should then be tested by an assay that can differentiate antibodies to HIV-1 from those directed against HIV-2 (Multispot HIV-1/HIV-2 Rapid Test; Bio-Rad Laboratories); samples that are nonreactive on the initial immunoassay do not require further testing and can be reported as negative (no evidence of HIV infection). Detection of antibodies specific for HIV-1 or HIV-2 is evidence of established infection with HIV-1, HIV-2, or both,

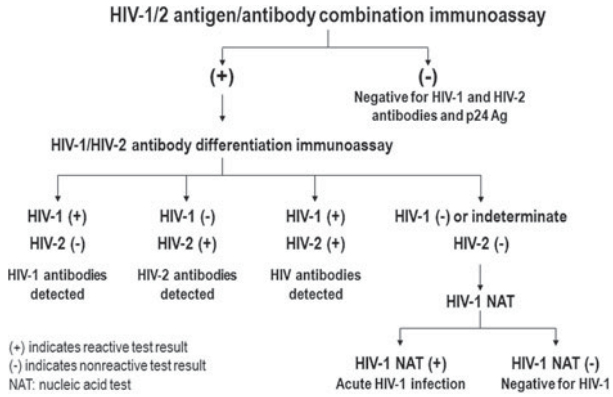

FIGURE 11 Testing algorithm for HIV diagnosis (228).

depending on the pattern of reactivity. Samples that yield negative or indeterminate results in the HIV-1/HIV-2 antibody differentiation assays should be tested by an HIV-1 nucleic acid test (i.e., plasma HIV-1 RNA assay). A positive HIV-1 RNA test, along with a negative or indeterminate test for HIV-1- or HIV-2-specific antibodies, is evidence of acute HIV-1 infection; a negative result indicates a false-positive reaction on the initial combination immunoassay. (There are currently no FDA-approved assays to diagnose acute HIV-2 infection.)

In addition to the standard HIV immunoassay, rapid diagnostic tests based on red cell or particle agglutination, as well as dot-blot assays, have been developed. The simplicity and wide range of operating temperature for some of these rapid tests make them particularly well-suited for point-of-care testing. Field testing of a rapid test approved for detection of HIV-1/-2 antibodies in whole blood, plasma, and oral fluids (OraQuick Advance; OraSure Technologies) found a sensitivity of 99.7% in whole blood and 99.1% in oral fluid, with specificities of 99.9% and 99.6%, respectively (361). However, the occurrence of 16 false positives in one study resulted in a specificity of only 99.0%. For this reason, a positive result by a rapid HIV antibody test should be confirmed using the algorithm described above, beginning with an antigen/antibody combination immunoassay.

Diagnosis of HIV-1 Infection in Neonates and Infants

Placental transfer of HIV-1 IgG antibodies from infected mothers to their fetuses poses special challenges to the early diagnosis of HIV-1 in infants and neonates. All infants born of HIV-1-infected mothers are initially HIV-1-seropositive (322, 323). Titers of maternal IgG decay over 12 to 15 months. Persistence of HIV-1 antibodies beyond 15 months is therefore considered diagnostic of infection in the infant. Infection with HIV-1 can reasonably be excluded by two or more negative HIV-1 serologies performed at least one month apart in infants older than six months of age.

Early identification of infected infants is essential to maximize the potential benefits of antiretroviral and prophylactic therapies, while minimizing exposure of uninfected infants to the potential toxicities of these therapies. Consequently, the diagnosis of HIV-1 infection in infants depends on virologic assays (e.g., virus culture or DNA or RNA PCR). Current guidelines recommend testing infants born to HIV-infected mothers at age 48 hours, at age one to two months, and at age three to six months. A positive test suggests the possibility of HIV-1 infection, which should be confirmed by a second test as soon as possible. DNA-PCR testing is positive in approximately 40% of infected children by age 48 hours and in 93% of infected children by age 14 days (362). Virus culture has similar sensitivity and specificity as DNA-PCR tests. Plasma HIV-1 RNA testing detected infection in significantly more infected infants at birth and at six weeks of age than did either HIV-1 culture or DNA PCR (363). Serum p24 antigen assays are less sensitive than other virologic assays for diagnosis of HIV-1 infection in infants and have a high false-positive rate in infants younger than one month of age (364).

HIV-1 Drug Resistance Testing

HIV-1 resistance to antiretroviral agents can be assessed by genotypic and phenotypic assays (365, 366) (see also Chapter 15). Advances in molecular diagnostic techniques have made these tests routinely available to clinicians. Genotypic assays determine the sequence of protease (PR), reverse transcriptase (RT), or integrase (IN) coding regions of the *pol* gene, whereas phenotypic assays determine susceptibility of a patient's virus to specific drugs in an infected cell culture system. The two kinds of assays provide complementary information. Each approach has distinct advantages and disadvantages, and both types of assays share certain limitations.

Resistance assays for HIV-1 depend upon initial amplification of the coding sequences of selected HIV genes targeted by drugs from plasma viral RNA by reverse transcription followed by PCR. For genotypic analysis, the amplicons are then subjected to automated DNA sequencing, probed by hybridization-based assays, or tested by point-mutation assay. Automated sequencing provides the most comprehensive data regarding genotypic changes associated with drug resistance. Phenotypic tests are performed by generating recombinant viruses or pseudoviruses using sequences from patient samples together with a molecular clone of HIV-1 deleted in the gene of interest. The resulting recombinant viruses (or pseudoviruses) share a common genetic backbone but express PR, RT, IN, or Env from the patient's virus. This approach eliminates many of the problems associated with older assays performed with primary virus isolates in peripheral-blood mononuclear cells, which had considerable interassay variation.

A limitation shared by currently available drug-resistance assays is their relative insensitivity to the presence of minority species in the virus population. Resistant variants of HIV-1 generally are not detected by most genotypic and phenotypic assays until they constitute more than 20 to 30% of the quasispecies. In addition, because of technical limitations in the RT-PCR step required to amplify viral sequences from plasma HIV-1 RNA, specimens with HIV-1 RNA levels less than 500 copies/ml may fail to generate a result.

Genotypic assays have the relative advantage of being faster and easier to perform, resulting in quicker turnaround times and lower cost than phenotypic assays. In addition, sentinel mutations may be detectable by genotypic assay before a shift in drug susceptibility becomes apparent. A major limitation of genotypic assays is the difficulty in predicting the consequences of mutational interactions on phenotype. Likewise, the extent of cross-resistance among drugs within a class (e.g., protease inhibitors) can be difficult to predict on the basis of genotype alone. The Stanford University HIV Drug Resistance Database (http://hivdb.stanford.edu/) offers useful guidance and a variety of tools for the interpretation of genotypic resistance tests. The

application of emerging "next-generation" sequencing technologies may provide the capacity to generate HIV-1 genotypes with higher throughput and at lower cost. These technologies may make detection of minority variants feasible in clinically useful tests.

Phenotypic assays have the advantage of providing susceptibility data in a format that is familiar to most clinicians (i.e., 50% inhibitory concentration [IC_{50}] or fold-resistance), as well as the capacity to determine drug susceptibility, even if the genetic basis of resistance to a particular drug is uncertain, and the net effect of different mutations on drug susceptibility and cross-resistance.

Strong correlations exist between genotype or phenotype at the time of regimen switch and the subsequent virologic response to salvage therapy. One meta-analysis showed that the risk of virologic failure was reduced by 30 to 50% for each drug in the salvage regimen to which the virus was susceptible, as predicted by the resistance test employed (367). In randomized trials (368) selection of a salvage regimen with the assistance of resistance testing resulted in significantly greater decreases of plasma HIV-1 RNA or a greater proportion of patients achieving a plasma HIV-1 RNA level below the limit of detection, although follow-up in most studies was brief.

Resistance testing is recommended to help guide the choice of new regimens after treatment failure and in pregnant women (369). In addition, resistance testing should be performed prior to initiating therapy in treatment-naïve patients, particularly in areas with a high prevalence of drug resistance in recently transmitted viruses. Testing is also advisable in patients with primary HIV infection, but treatment should not be delayed while awaiting results. Because resistant variants can be replaced by wild-type virus within weeks of discontinuing treatment, resistance testing is most accurate when the sample is obtained while the patient is still on the failing antiretroviral regimen. If high-level resistance to a drug is detected, that drug is unlikely to be useful in a treatment regimen. Failure to detect resistance to a previously-used drug does not necessarily imply activity of that drug, however, for reasons cited above. Because sample mix-ups and PCR contamination can lead to reporting of erroneous results, clinicians should not hesitate to repeat a resistance test if the results are markedly discordant with the treatment history or results of viral load and CD4 testing.

PREVENTION

General

The mechanisms and risks for transmission of HIV are clearly defined. Virus in blood and genital secretions accounts for practically all infections. By excluding blood donors at risk for infection and by testing blood products for HIV, transmission by this route can be virtually eliminated. Unless and until an effective vaccine is available, the combined implementation of the other prevention strategies that have been proven to be effective remain the best opportunity to reduce expansion of the pandemic (370).

The major causes of adult transmission of HIV, sexual intercourse and injection-drug use, represent two extremely strong biologic drives. Transmission can be significantly diminished, if not avoided, by monogamous sex with a known uninfected partner, use of barrier contraceptives, and not sharing drug paraphernalia. Many individuals have successfully modified their risk behaviors to prevent infection. For example in the San Francisco homosexual men's cohort, HIV spread early and rapidly between 1978 and 1984. When the mechanism of its transmission was recognized and behavior changed as a result, new infections in this cohort diminished remarkably (371). Unfortunately, new generations of young men who have sex with men are often ignoring these stark lessons; this risk behavior also contributes significantly to transmission in developing countries (372). Educational programs regarding condom use have appeared to have dampened rates of transmission in some locales. Needle-exchange programs to reduce sharing of contaminated paraphernalia by intravenous drug users reduces HIV transmission, but in many places in the United States, former Soviet Republics, and Southeast Asia, socioeconomic or political considerations thwart implementation of this intervention (373, 374). One persisting obstacle to any effective intervention, whether treatment or prevention, is the substantial proportion of populations not yet identified as infected or at risk (375, 376).

Preexposure Prophylaxis (PREP)

The administration of antiretroviral drugs to uninfected individuals at risk of transmission to prevent acquisition of HIV has rapidly progressed from an intense area of investigation to an approved indication for the use of a fixed-dose combination of daily oral tenofovir disoproxil fumarate and emtricitabine (TDF/FTC). The efficacy of this combination or TDF alone has been demonstrated in clinical trials with MSM and transgender women (377), with serodiscordant heterosexual couples and young heterosexual men and women in Africa (378, 379), and with injecting drug users (380). Efficacy was not shown in two clinical trials with heterosexual women in Africa (381, 382). The prime determinant of reduced efficacy in the successful trials and the lack of efficacy in the unsuccessful trials appears to be poor adherence. Animal models have supported both the observations about efficacy and the requirement for adequate drug levels in blood and genital and rectal mucosal tissues. Injectable antiretroviral drugs with half-lives of weeks to months, such as cabotegravir and long-acting rilpiverine, confer prolonged protection in a macaque model and are undergoing human trials (383).

Many healthcare providers and subjects at risk have been reticent to implement PrEP more actively. Obstacles to uptake include drug cost, the stigma of taking antiretroviral drugs, discomfort with new approaches, poor appreciation by either subjects or providers of the level of risk for infection, and concern about encouraging risk compensation. Efforts to document increased behavioral disinhibition with any prevention strategy have not provided documentation for this concern. Drug resistance can develop if PrEP is administered shortly after transmission has occurred (377) or during periods of suboptimal drug levels resulting from poor adherence. Such situations have occurred, but not commonly (384). The search for additional drugs and the conduct of additional clinical studies remain intense areas of investigation.

One special type of PrEP is topical prevention for women with vaginal preparations. In many cultural and social situations, women who have limited choice regarding sexual encounters could utilize effective prophylaxis without the need of a partner's consent. The initial high priority in this area was to develop a vaginal microbicide effective against HIV (385). Ideally the microbicide would be active against other sexually-transmitted infections as well, since they increase transmission rates of HIV by producing mucosal ulceration and local inflammation (175). Unfortunately the

similarity between the retroviral envelope and the host cell membrane provided a challenge to identify inactivating agents with selective activity. In phase 3 trials, the topical microbicides nonoxynol-9 and cellulose sulfate both increased transmission of HIV, presumably by disrupting the integrity of the natural mucosal barrier (386), while Carraguard fared no better than placebo. Consequently, the focus shifted to the use of safe and effective antiretroviral drugs. The self-administration of 1% tenofovir gel by South African women conferred protection against HIV (and herpes simplex virus), once again with the degree of success correlating with the level of adherence (387). One clinical trial with both an oral and a vaginal gel arm showed no efficacy, attributable to poor adherence (382). Additional drugs and formulations, including vaginal rings that slowly release maraviroc or a nonnucleoside reverse transcriptase inhibitor, davirapine, are being actively investigated. Similarly, formulations for gels to be used intrarectally for prophylaxis are being investigated. More thorough reviews of all aspects of PrEP are available (386, 388–390).

Male Circumcision

Male circumcision has been shown in randomized controlled trials to reduce the transmission of HIV, herpes simplex virus, human papillomavirus, and genital ulcer disease in men, and to reduce human papillomavirus, genital ulcer disease, bacterial vaginosis, and trichomoniasis in their female partners (391). Randomized trials in uninfected men have shown a 50 to 60% protective effect of circumcision against heterosexually-acquired HIV (392–394). This intervention is less effective than condom use but does not require adherence with each sex act and presumably remains effective for life. Thus, its cost-effectiveness has prompted many implementation initiatives and has been supported by many low- and middle-income countries (395). These studies have consistently confirmed the benefits in heterosexual men with substantial cost-effectiveness. The benefits in men who have sex with men remain to be documented. The implementation efforts in Africa had provided circumcision to over six million males by 2015 with increasing implementation in many countries.

Passive Immunoprophylaxis

With the identification of more potent and broadly-reactive neutralizing monoclonal antibodies, initiatives to assess these alone or in combination for prevention or treatment are in progress (396). One critical challenge has been the difficulty in eliciting potent broadly-neutralizing antibody by natural infection or candidate immunogens. The administration of monoclonal antibodies or polyclonal sera that provide neutralizing activity against the challenge virus can confer protection from infection in both murine and rhesus macaque models (397, 398). The administration of neutralizing monoclonal antibodies, alone or in combination, suppressed viremia in rhesus macaques and delayed the reemergence of detectable viremia in humans after suppressive antiretroviral therapy was withdrawn (399–401). The viruses that emerged were neutralization-escape variants to the monoclonal antibody 2G12, indicating that the selective pressure conferred by that antibody contributed to the delayed emergence of replicating virus. The development of more promising antibodies (402) and insights into engineering monoclonal antibodies with better effector functions and longer half-lives (403, 404) have renewed the energy being put into the development of promising biologicals to administer to humans and into clinical trials to apply these for prevention and treatment (405, 406).

Prophylaxis

Postexposure prophylaxis has proven effective for those exposed either occupationally or during sexual activity (407). Approximately one in 300 healthcare providers becomes infected with HIV after percutaneous exposure with a needle used on a seropositive patient (178). With the inoculum size in such transmissions probably representing close to one human infectious dose, prompt administration of chemoprophylaxis may be beneficial. Because of the low rate of transmission, controlled randomized trials are not feasible, and decisions must be based on judgment, retrospective studies, and animal models. A retrospective analysis of the use of zidovudine for postexposure prophylaxis of healthcare workers after percutaneous exposure to infected blood suggested an 80% reduction in risk of seroconversion (408). Prevention of needlestick transmission is best accomplished by prevention of needlesticks. This requires care and attention by healthcare workers during injection and phlebotomy procedures, use of gloves, proper disposal of sharp instruments, and the use of needles with guards and other devices designed to minimize risk.

The issue of risk to patients from infected providers has raised much controversy. The mechanism of transmission in one outbreak associated with a Florida dentist remains unexplained (409). Nevertheless, routine healthcare provided by HIV-infected providers poses no measurable risk of transmission, and effective antiretroviral treatment virtually eliminates any risk.

Considerations similar to those for healthcare providers apply to postexposure prophylaxis following sexual exposure due to rape, broken condom, or other unprotected consensual or nonconsensual sex (410). The decisions regarding implementation and regimen significantly overlap with those described for occupational exposure.

Extrapolation from other applications of chemoprophylaxis for infections would argue that the earlier the administration and the more potent the regimen, the greater the likelihood that the prophylaxis will work. Because many HIV-infected subjects have been infected with transmitted drug-resistant virus or they have been treated with antiretrovirals and thus may harbor drug-resistant virus, guidelines recommend tenofovir disoproxil fumarate, either lamivudine or emtricitabine, and a third agent (efavirenz, a boosted protease inhibitor or an integrase inhibitor) (410). Because of drug tolerability, lack of drug interactions, and risks of drug resistance in the source subject, integrase inhibitors have become the preferred third component. The recommended 28-day duration of prophylaxis is also based on judgment and consensus rather than empirical data, although studies in the macaque model indicated equivalent benefit with shorter durations. Early initiation of prophylaxis, potent regimens, and adherence are almost certainly more important than duration of prophylaxis.

Treatment as Prevention

The suppression of HIV in blood and genital secretions with antiretroviral therapy is associated with the reduction, if not the elimination, of the risk of transmissibility (411, 412). The approach, termed treatment-as-prevention, has been well documented in studies of heterosexual transmission among serodiscordant couples (411, 413) but likely applies to others at risk of transmitting, including MSM and injection-

drug users. On an epidemiologic basis, reductions in HIV-1 incidence correlate with relative saturation density of coverage of antiretroviral therapy (414–416). Certainly the recommendations to benefit individuals with universal treatment in resource-rich countries (347) and expanded treatment in low- and middle-income countries (417) is compatible with the public health benefits of diminishing HIV incidence with extensive treatment of populations. The challenge remains that both the individual and public health benefits of expanded treatment have been constrained by insufficient screening, access to care, and resources for healthcare and drugs.

Mother-to-Child Transmission

Maternal-fetal transmission represents a unique opportunity for prevention because risks can be readily ascertained with reasonable lead times and effective interventions have been identified. By 2013, 3.2 million children were living with HIV infection with over 650 children newly infected daily, one-half of whom would die by their second birthday without treatment (418). These numbers reflect diminishing numbers of new infections because of prevention of mother to child transmission but increasing numbers of children living with HIV because of the survival benefits of antiretroviral treatment. Maternal-fetal transmission occurs antepartum by transplacental transmission, during delivery, and via breastfeeding (419). Thirteen to 40 percent of untreated HIV-infected pregnant women transmit infection to their newborn infants (419). Perinatal administration of zidovudine alone to treatment-naïve pregnant women with more than 200 CD4 cells/μl blood and to their newborns reduced transmission from 25.5% to 8.3% (157). With these perinatal interventions, the protection is primarily conferred as postexposure prophylaxis in the newborn (420). Implementation of strategies to prevent maternal-fetal transmission in low- and middle-income countries began with the very low-cost single-dose nevirapine, which led to high levels of resistance in both mother and child. This led to increasingly more effective combination perinatal regimens (421). The current strategy is to offer combination antiretroviral treatment to the pregnant mother at diagnosis of infection, regardless of CD4 cell count or clinical stage, and to maintain treatment for life. This last strategy, called option B+, is the WHO recommended approach (422), having the advantage of both providing the most effective (and cost-effective) (423) approach to preventing transmission to the newborn and maintaining the health of the mother. Treating the mother permits breastfeeding with greatly-diminished risk and results in survival and quality of life benefits for the mother and for both her infected and uninfected children. Challenges remain including the testing of mothers with unknown HIV status, adherence, and retention in care (421, 424). The expensive but potentially effective approach of prophylaxis with passively administered monoclonal neutralizing antibodies as discussed above has entered clinical trials (425).

Vaccines

The prevention strategies described above can each reduce transmission; however, a relatively cost-effective intervention with the promise to have a major impact on incidence almost certainly requires a vaccine. The challenges are substantial, however (Table 4). Ideally an HIV vaccine would induce a sustained high level of broadly cross-reactive neutralizing antibodies, which is the primary correlate of protection of most effective viral vaccines (426). It is quite possible that other nonneutralizing activities of envelope-specific antibody (e.g., antibody-mediated cellular cytotoxicity) or CD8 CTL responses would contribute to vaccine efficacy (426–428). However, more than two decades of efforts to design an effective vaccine have only confirmed that approaches that have worked for so many effective vaccines are insufficient to address HIV.

TABLE 4 Obstacles to an effective vaccine for HIV

1. The immune responses conferring protection and mediating viral clearance and the viral antigens that elicit these responses are not defined.
2. HIV displays great antigenic diversity among individuals.
3. HIV mutates readily to generate escape mutants and genetic diversity within individuals.
4. The HIV envelope glycoprotein is heavily glycosylated, which shields many potential epitopes.
5. Mucosal immunity may be needed.
6. Enhancing or blocking antibodies may exist.
7. The viral genome integrates into the host cell chromosome.
8. The major target organ of HIV is the immune system.
9. No inexpensive, simple animal models exist.
10. Most effective viral vaccine vectors are subject to pre-existing antivector immunity and elicit immunity that compromises re-use.
11. High-titer, broadly-reactive neutralizing antibody responses do not occur within a year in any subject or over many years in most subjects with natural infection. Such antibodies require extensive affinity maturation.
12. No immunogen has been designed that elicits neutralizing antibody that is not strain-specific or that has a high titer.

Studies in macaques have indicated the protective value of passively-administered neutralizing antibody (398). However, naturally-occurring neutralizing antibody responses to infection are of low titer and of restricted cross-reactivity in humans, and most antibodies to the HIV envelope exhibit poor neutralizing activity. Neutralizing antibody develops within two to three months after infection; however, this antibody is strain-specific, and more broadly-reactive neutralizing-antibody responses develop only over years and only in a subset of individuals (28). Neutralizing-antibody responses to candidate vaccines have been weak and restricted to laboratory-adapted strains rather than primary isolates (397). Modified envelope-protein preparations have not yet been designed that elicit neutralizing antibody to heterologous strains. Thus, although neutralizing antibody comprises the basis of most protective viral vaccines (426), new basic insights will be needed to elicit protective humoral responses to HIV infection.

Extensive efforts have been employed to induce HIV-specific CTL. Historically this has been done using live, attenuated virus to express viral antigen in the context of autologous HLA-restricted presentation of epitopes. The use of live, attenuated HIV has been complicated by concerns over the balance between immunogenicity and attenuation (429), which may be unpredictable and highly variable in humans with their heterogeneous genetics, ages, health, and nutrition. Efforts to induce CTL have thus focused on the expression of HIV antigens either with "naked" DNA or with viral or bacterial vectors. Virtually every available vector has been studied as a candidate, but the most extensive studies with primate and human trials have utilized

poxviruses (vaccinia and avian) and adenoviruses. The literature in rhesus macaque models is replete with negative or borderline levels of protection. Substantial CTL responses after DNA priming with adenovirus or poxvirus boosting or after sequential vector immunizations have resulted in reductions in HIV RNA "set point" and rates of progression in rhesus macaque models of SIV or SHIV infection; however, the effect is limited in magnitude and duration and is usually overcome by viral immune-escape mutations (430, 431). Investigation of approaches to elicit effective cell-mediated immune responses are focusing on the design of viral vectors that are effective and resistant to pre-existing immunity in the population, as well as the design of expression construct that will elicit effective responses to protective epitopes. Since no T-cell-based vaccine has been shown to confer protection in humans, evidence is needed to show whether this approach can replace or enhance an antibody-based approach. One innovative strategy that appears to have a qualitatively superior level of protection in the rhesus macaque model is a vaccine construct in an attenuated CMV vector, which has been shown to elicit a range of T-cell responses not characteristic of the usual class I HLA cell-mediated immune responses (432).

Dozens of candidate HIV vaccines comprised of protein constructs and gene constructs delivered by naked DNA or an array of microbial vectors have been tested in phase I and II clinical trials (433). In general both neutralizing antibody and cell-mediated immune responses have been lower and more restricted in breadth than natural infection, which in itself is poorly protective against superinfection (434, 435). Larger randomized placebo-controlled trials have also provided limited encouragement. Gp120 glycoprotein vaccines elicited some neutralizing antibody against the vaccine strain, but did not elicit a broad response and conferred no protection compared to placebo (436–438). Three trials of vaccines based on adenovirus-5 vectors showed no efficacy (439–441) with the Step trial suggesting an increased risk of transmission in uncircumcised men with high-baseline antibody levels to adenovirus 5 (439). The large RV 144 trial used a canarypox vector expressing a CRF01_AE *env* as a priming immunization, followed by a boost with bivalent B and E monovalent gp120 glycoproteins in alum adjuvant, and conferred a 31% rate of protection with up to 42 months of follow-up (442). The components of the vaccine that most contributed to this activity, to the durability of efficacy, and to the mechanism of this low level of protection remain important issues for future vaccine development. Analyses to find correlates of this low level of protection have suggested that antibody responses to V1/V2, IgG3 responses, and Fc function, possibly related to antibody-mediated cellular cytotoxicity, may have been related to protection (443). Variants of this approach have been designed for studies to attempt to confirm efficacy in other populations at risk for different subtypes of HIV-1.

The development of innovative approaches to the design of an effective HIV vaccine remains a high priority for a highly-effective prevention strategy. The identification of a series of neutralizing monoclonal antibodies that are much more potent and broadly reactive than previously recognized, as described above, has prompted investigations into the process immunoglobulin maturation in B cells from germ line to protective immunoglobulin responses (444). Antibodies are also being studied for efficacy mediated by activities conferred by nonneutralizing responses and via functions of the Fc portion of the molecule (404, 445). Efforts have intensified to design immunogens that can elicit more than a strain-specific antibody response. A more innovative approach is to deliver antibodies or related constructs intramuscularly in an adeno-associated viral vector that can generate high levels of broadly neutralizing antibody constructs for years (446, 447).

TREATMENT

The improvement in AIDS mortality statistics reflects the introduction of combination antiretroviral treatment regimens first in Western Europe and the Americas and later in low- and middle-income countries (Fig. 12) (448–450). Opportunistic disease has been reversed and prevented. Healthcare costs have diminished. Many ill and disabled patients have returned to normal and functional life styles. This dramatic impact does come with costs—the expense, inconvenience, and toxicity of antiretroviral therapy. These costs and the benefits of treatment initially created a tension in the decision-making process regarding when to initiate

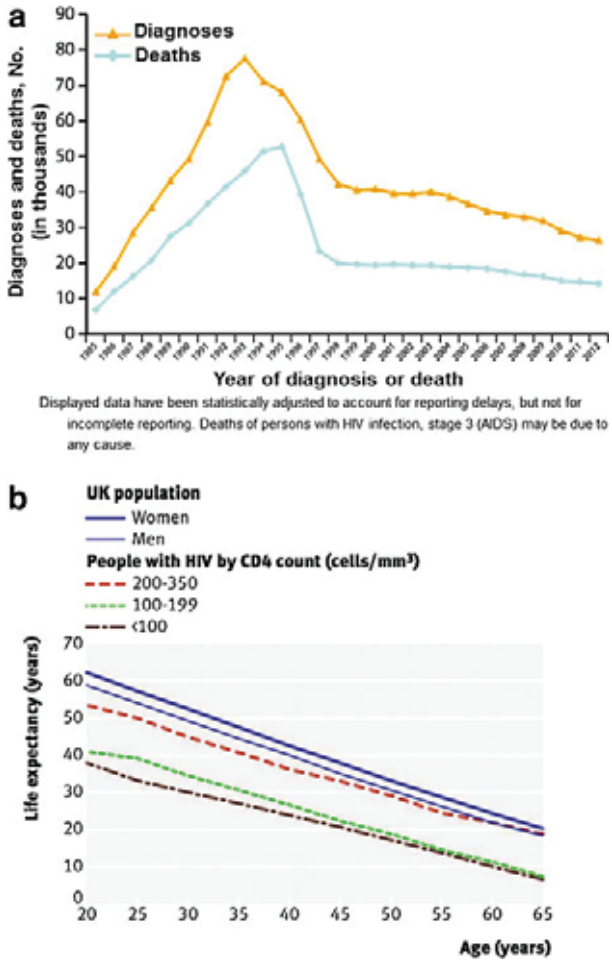

FIGURE 12 a) Estimated incidence of AIDS and deaths of adults/adolescents with AIDS in the United States during the period 1985–2005. Number of deaths is adjusted for reporting delays. b) Estimated proportion of persons surviving with AIDS in the US by year of diagnosis. (Both figures adapted from the CDC; http://www.cdc.gov/hiv/.) With new infections continuing unabated and with survival increasing as a result of improving treatment, one consequence is the progressive accumulation of persons living with HIV infection.

therapy. With the development of drugs that can be taken for decades with low toxicity, as well as fixed-dose combinations that require only one pill daily, the treatment guidelines have expanded to recommend treatment for all HIV-infected individuals in resource-rich countries (347, 451) and all individuals in low- and middle-income countries with CD4 cell counts below 500 cells or who are pregnant or have active tuberculosis, chronic hepatitis B, or a serodiscordant partner (417). Earlier initiation of treatment prevents complications and death, preserves immune function, reduces the size of the latent HIV reservoir, and prevents transmission.

Because of the rapid advances in treatment, for specifics regarding the use of antiretroviral drugs and the management of HIV infection, the reader is advised to refer to the updated versions of the guidelines referenced previously. This chapter, rather than provide a manual for chemotherapy, will summarize the principles and challenges of the treatment of HIV infection. The antiretroviral drugs, including their mechanisms of action, pharmacology, and toxicities are summarized in Chapter 11.

Virologic and Immunologic Principles Underlying Antiretroviral Chemotherapy

The primary goal in the management of antiviral therapy for the HIV-infected patient is to achieve prolonged suppression of viral replication. While a small subset of individuals, for reasons attributable to the genetic composition of the virus or immunologic responses, may survive years to decades without an increase in viral burden (452, 453), HIV infection in most individuals leads to increasing levels of HIV, decline in CD4 cells, and death.

As discussed in the Pathogenesis section, the turnover of virus particles in the body is tremendous, clearance of virus from the plasma is rapid (minutes to hours), and the clearance rate constant varies little among individuals and different stages of disease. The steady-state levels of HIV RNA in the blood are thus determined by the rate of virus production. These rates of production are a function of the number of infected lymphocytes in the lymphoid tissue (454, 455). The rate of decline of CD4 lymphocytes is thus directly related to the steady-state level of plasma HIV RNA. The higher the RNA levels, the faster the loss of CD4 cells, and the shorter the duration of HIV infection before death (210). Since the CD4 count determines the risk of disease and death, and the level of HIV RNA determines the rate of CD4 cell decline, these values are routinely used clinically to assess clinical status and response to chemotherapy.

When potent combination therapy is effectively administered, levels of HIV RNA in plasma and infected cells in lymphoid tissue rapidly decrease (Fig. 9 and Fig. 13a). Failure to reduce plasma HIV RNA levels to below the limits of detection of 20 to 50 copies/ml with the currently-available assays indicates inadequate suppression and a risk for the outgrowth of resistant virus. In patients sustaining suppression below this level (Fig. 13b), many will sustain steady-state levels of 1 to 40 copies HIV RNA/ml, which is not associated with clinical progression or with viral evolution (241). The relative contributions of smoldering replication, release of virions from activated latently-infected cells, and persistently-infected long-lived cells have not been well delineated.

Compartments and Reservoirs

The treatment of HIV is complicated by the existence of tissue compartments and cellular reservoirs. Although there

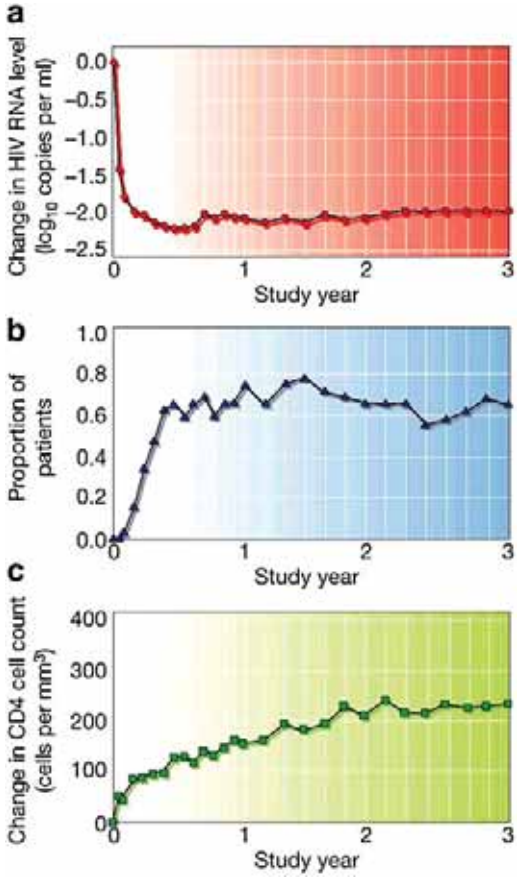

FIGURE 13 Three-year treatment with indinavir, zidovudine, and lamivudine. a. Median changes in serum HIV RNA level (Amplicor assay with quantification limit of 500 copies per milliliter) from baseline. b. Proportions of patients with serum HIV RNA levels less than 50 copies per milliliter (ultradirect assay). c. Median changes in CD4 cell count from baseline. The number for contributing patients in each trial and at each time point was between 30 and 33. Details regarding the study and analyses are published (464). Adapted from reference (484) with permission.

is trafficking between the blood and CNS, much virus in the CNS evolves independently (286,456–458). Similar observations have been made with virus in semen (459–461). Drug penetration into these compartments differs from the circulation and lymphoid tissue and varies with each drug. Latently-infected CD4 lymphocytes represent a small fraction of infected cells during active infection, but like immunological memory, persist for life (229). Such cells survive, archiving virus that can be drug-resistant, and reemerge and propagate after the withdrawal of chemotherapy.

Immunological Restoration with Antiretroviral Therapy

The immunologic consequences of suppressing virus replication are dramatic (Fig. 13c). The increase in CD4 lymphocyte numbers has two phases. In the first month or two the increase is often large (20 to 100 cells per μL blood) (462–465). The magnitude is proportional to the steady-state HIV RNA levels, which drive the level of generalized activation of the immune system. The normal distribution of

lymphocytes is 2% in the circulation and 98% in the lymphoid tissues. With the immune activation of HIV infection, the distribution shifts to 1% and 99% (454, 466). Therapy largely corrects this shift and results in redistribution of mostly CD45RO$^+$ memory T cells from the lymphoid tissue back to the circulation (465, 466). Production of new cells, mostly of the CD45RA-naive phenotype, is generated both by bone marrow and by restored thymic mass and function in younger individuals (247, 467).

It is the restoration of immune function that has transformed the natural history of AIDS. Both CD4 and CD8 T-cell responses to recall antigens are regenerated (462, 468). Persistent opportunistic infections are often resolved. Occasionally subclinical chronic infections, with mycobacterial or cytomegalovirus infections, for example, are manifested when a restored immune response produces a local inflammatory reaction, termed the Immune Reconstitution Inflammatory Syndrome (IRIS) (469, 470). Patient care has been transformed with the ability to withdraw prophylactic or suppressive chemotherapy for pneumocystis, toxoplasma, cytomegalovirus, *Mycobacterium avium* complex, Leishmania, cryptococcus, and candidal infections, which had previously been lifelong commitments.

Drug Resistance in Antiretroviral Chemotherapy

Antiretroviral drugs select for the emergence of drug-resistant viral variants. These mutations, and their impact on phenotype and treatment, have been well-described (366, 369, 471), but it is important to note that the speed, magnitude, and clinical impact of the emergence of resistance differ among antiretrovirals. The likelihood that resistant mutants will emerge is a function of at least four factors: (1) the viral mutation frequency, (2) the intrinsic mutability of the viral target site with respect to a specific antiviral, (3) the selective pressure of the antiviral drug, and (4) the magnitude and rate of virus replication.

For single-stranded RNA viruses, whose genomic replication lacks a proofreading mechanism, the mutation frequencies are approximately 10^{-5} per nucleotide per replication cycle or approximately 1 mutation for every progeny genome (472, 473). Some mutations at a single nucleotide will result in greater than 100-fold reductions in susceptibility, for example, to lamivudine or nonnucleoside reverse-transcriptase inhibitors (366). For many nucleosides and protease inhibitors, high-level resistance requires the cumulative acquisition of multiple mutations.

With regard to the selective pressure of the antiviral drug, one definition of an antiviral drug is a compound that confers sufficient selective pressure on virus replication to select for drug-resistant mutants. With increasing drug exposure, the selective pressure on the replicating virus population increases to promote the more rapid emergence of drug-resistant mutants. For example, higher doses of zidovudine or of ritonavir monotherapy tend to select for drug-resistant virus more readily than do lower doses (474, 475).

With current drug treatment, this relationship is most apparent when patients with suboptimal adherence develop resistance more readily than those with very poor adherence. Increasing selective pressure for resistant mutants increases the likelihood that such mutants will arise as long as significant levels of virus replication persist (Fig. 14). As antiviral drug activity increases still more, the amount of virus replication diminishes to the point where the likelihood of emergence of resistance begins to diminish, and becomes nil when virus replication is completely inhibited. No evolution of an HIV nucleotide sequence can be discerned for over a decade of fully-suppressive antiretroviral therapy. Thus the ultimate goal of chemotherapy for HIV is to identify drug regimens that completely inhibit virus replication.

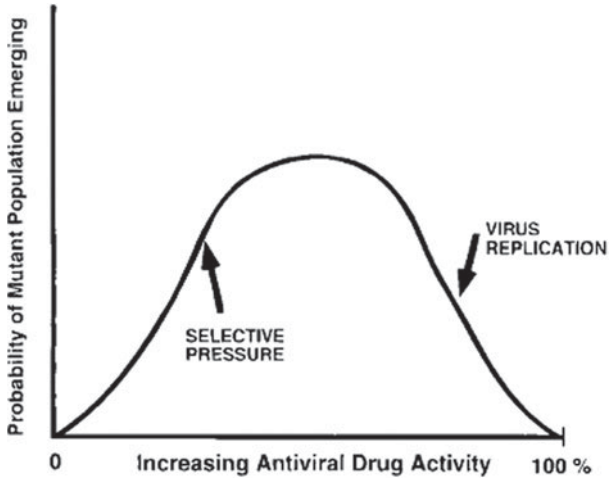

FIGURE 14 Hypothetical impact of antiviral drug activity upon the probability of the emergence of drug resistance. From reference (485) with permission.

The magnitude and rate of replication of the virus population have major consequences on the likelihood of emergence of resistant mutants. Approximately 10 billion (10^{10}) HIV-1 virions are generated daily (106), and approximately one mutation is generated for each new genome of 9200 nucleotides (473). Thus, genomes with each possible mutation, as well as many with double mutations, should be generated daily. Incompletely-suppressed viral replication, with drug regimens sufficient to exert selective pressure, drive the evolution and fixation of drug-resistant virus at a rate Darwin himself never imagined. Moreover, drug-resistant virus is readily archived in latently-infected cells to confound treatment modifications for the remainder of the patient's life (227, 228).

Impact of Resistance on Treatment

As resistance mutations accumulate, drug susceptibility diminishes, progressively reducing the potency of components of combination antiretroviral regimens. Continued replication in the presence of drug selects for even greater levels of resistance to each administered drug and progressive cross-resistance to drugs of the same class. Thus, impotent regimens, suboptimal adherence, pharmacologic hurdles, and ineffectively-treated compartments permit the emergence of resistant virus. Its emergence drives a vicious cycle of treatment failure and yet more difficult treatment challenges. Regimens for patients failing treatment with resistant virus are constrained by more limited options but must still contend with the same obstacles of adherence, pharmacology, and tolerability that challenged the first regimen.

Resistant virus in genital secretions, blood, or milk can be transmitted during sexual activity, needle-sharing, childbirth, or nursing (294). With the more widespread use of nucleoside-only regimens and then early three-drug combination regimens in the 1990s, the rates of transmission of drug-resistant virus increased dramatically, with up to 20% of primary infections due to drug-resistant virus, many of which exhibited resistance to multiple classes of drugs (294, 476). Patients with such resistance are more likely to fail their first treatment regimen. With more effective and tolerable

regimens requiring one or a few pills, the rates of both acquired- and transmitted-resistance have plateaued or even diminished in resource-rich countries. In low- and middle-income countries, where the first decade of the rollout of antiretroviral drugs included thymidine analogues, the increasing rates of acquired- and transmitted-resistance recapitulated the earlier experience in more developed countries (477).

Resistance Testing to Manage Patients

The accumulation of drug resistance due to treatment failure and transmission raises challenges to the effective treatment of individuals and to public health. As discussed above, the use of drug-resistance testing has been incorporated into standard HIV care (365, 366, 471). Drug-resistance assays can help determine which drugs will not work, thereby diminishing cost, toxicity, and inconvenience, and which drugs are most likely to be effective.

Antiretroviral Drugs

As of 2015, 30 antiretroviral drugs had been approved for use in the United States, and several more were in various stages of clinical development (Chapter 11). Although any function in a genetically-efficient organism is a candidate for an inhibitory drug, the currently-approved drugs are directed against reverse transcriptase, protease, integrase, and viral entry, with the reverse transcriptase inhibitors being classified as nucleosides or nonnucleosides.

The use of these drugs in the management of HIV-infected patients has developed into a remarkably complex specialty. These drugs must be used in various combinations of three or more drugs for optimal efficacy. Each drug has a complicated pharmacology that often interacts with other components of the regimen or with nonantiretroviral drugs. Treatment success is related to rigorous adherence. Initial regimens have become increasingly effective, tolerable, and convenient, with many regimens taken once daily. Regimens for treatment failure become increasingly complex and are attended by higher rates of undesirable side effects.

THE CHALLENGE OF CURING HIV

The latent HIV reservoir, which persists despite decades of effective antiretroviral therapy, represents an obstacle to cure. Antiretroviral therapy must be life-long, and withdrawal results in the resumption of high levels of virus replication within weeks. The "Berlin patient" underwent hematopoietic stem-cell transplantation with cells from a donor homozygous for the delta 32 deletion mutant of CCR5 (478). He has had no evidence of detectable virus or recrudescence of ongoing infection for more than seven years after discontinuing antiretroviral therapy; however, the relative contributions to this apparent cure of resistant donor cells, intensive chemotherapy for leukemia, and a vigorous graft versus host reaction cannot be dissected with a single anecdote.

The eradication of the latent HIV reservoir to achieve a cure has become an active area of investigation. Achieving a cure could relieve treatment fatigue, reduce drug costs, reduce drug toxicity, reduce drug resistance, reduce transmission, and reduce morbidity and mortality associated with viral persistence and immune activation (e.g., malignancies, cardiovascular disease, CNS impairment). The hurdles to success in achieving a cure include both the biology of latency and the current limitations in both knowledge and technology. The biological hurdles are several. One in a million or so CD4 T cells is latently-infected with replication-competent virus so the target is a very rare event in a sample. Two, the majority of the latent reservoir is in extra-circulatory tissues, which are difficult to access. Three, the mechanisms for establishment and maintenance of latency are cellular functions, which means that drug interventions will need to target host, rather than viral, targets, thus imposing greater risk of toxicity. Four, enhancing immune function to assist in clearing these latently-infected cells must address the fact that the immune system has often selected for escape mutants-to-host responses (278).

Similarly, the limitations in our knowledge and technology are several. One, do cells other than CD4 T lymphocytes, such as macrophages and microglial cells, represent a latent reservoir? Two, are there pharmacologic sanctuaries for latency like the central nervous system? Three, what are the mechanisms for the establishment and maintenance of latency that provide targets for candidate drug interventions? Four, will enhancement of the immune system with a therapeutic vaccine of monoclonal antibodies contribute to clearance of the latent reservoir? Five, can practical gene therapy approaches contribute to curative strategies? Six, how do we measure the latent reservoir and reductions in it by candidate interventions when latency occurs infrequently in cells and at most 1 to 2% of the reservoir exists in the blood?

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Colorado Tick Fever and Other Arthropod Borne Reoviridae

STEVEN YUKL AND JOSEPH K. WONG

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Among arthropod-borne viral infections, Colorado tick fever is second in incidence in North America only to West Nile fever (1). As with other viruses, evidence of infection by arthropod-borne reoviruses, including many newly identified viruses, as the cause of both human and veterinary disease continues to accumulate. This likely reflects advances in detection and diagnostic methods and perhaps also evolving demographic conditions that facilitate contact between human populations and the insect vectors that transmit these viral infections. The clinical importance of reoviruses will doubtless continue to change in the wake of the emergence of other arthropod borne infections such as Zika and dengue viruses.

COLORADO TICK FEVER

The earliest accounts of what was most likely Colorado tick fever emerged in the 19th century from settlers and mountaineers in the Rocky Mountains of North America who termed it, along with other febrile illnesses, "mountain fever" (2). By the time the rickettsial disease Rocky Mountain spotted fever (RMSF) was identified as a distinct entity in the first decade of the 20th century, cases of RMSF without rash were also being described, and some of these cases were likely Colorado tick fever (2–7). In 1930, Becker described the clinical manifestations and gave them the name Colorado tick fever (8). The clinical features and epidemiology were further characterized by Topping in 1940 (9). In 1944, Florio and colleagues reported the experimental transmission of the disease to animals and adult volunteers, thus establishing the presumptive viral etiology (10).

The development of viral cultures in chicken embryos and mice by Koprowski and Cox in the 1940s (11) and subsequent refinements in viral isolation and molecular biology permitted further characterization of Colorado tick fever virus (CTFV). Much is now known about its ecological niche and replication cycle in vertebrate and invertebrate hosts as well as the epidemiology, pathogenesis, and clinical course of infection in humans (5,12–17). A growing body of genetic sequence information has helped to delineate the relationship between CTFV and related viruses (18–24).

VIROLOGY

Classification

Three genera of arthropod-transmitted viral agents of medical importance are recognized within the family *Reoviridae*: *Coltivirus*, *Orbivirus* and *Seadornavirus* (South East Asian Dodeca RNA virus). The former is further classified within the subfamily *Spinareovirinae* (spiked or turreted; spina, Latin for spiked) while the latter two are classified with the *Sedoreovirinae* (non-spiked; sedo, Latin for smooth), although there is overlap in the morphological appearance of these genera by electron microscopy (EM) (25). Recognition of the fundamental difference in their genome structure (10 double-stranded RNA [dsRNA] segments in orbiviruses versus 12 dsRNA segments in coltiviruses and seadornaviruses) and other genetic attributes are responsible for the current classification (25–28). Species designations of members of each genus have been based on analysis of electropherotypes, RNA cross-hybridization assays, RNA sequence analysis, serologic reactivity, and the ability to reassort and produce viable progeny in co-infection experiments (25, 29). Phylogenetic analyses now provide a more precise picture of the relationships between different viral species (30) (Fig. 1).

CTFV is the type species of the genus Coltivirus (after Colorado tick fever). A number of other coltiviruses have been isolated and partially characterized and identified to species level. Eyach virus (EYAV) has been isolated from *Ixodes ricinus* and *Ixodes ventalloi* ticks in central Europe (31, 32). In a serologic survey of patients with meningoencephalitis and polyradiculitis in the former Czechoslovakia, 10% and 20%, respectively, had demonstrable antibody to EYAV, but a definite causal relationship remains to be proven (33). A coltivirus isolated from *Lepus californicus* hares in an area of northern California (outside the territory of *Dermacentor andersoni)* has been designated CTFV-Ca (formerly S6-14-03 virus) (23, 28, 34). Although this isolate has not been proven to cause human disease, it has been postulated to be responsible for Colorado tick fever-like human infection in California (35). A coltivirus dubbed Salmon River tick fever virus was isolated from a patient in Idaho with an illness similar to Colorado tick fever (35, 36). Whether this represents an antigenic variant of CTFV or a unique virus remains

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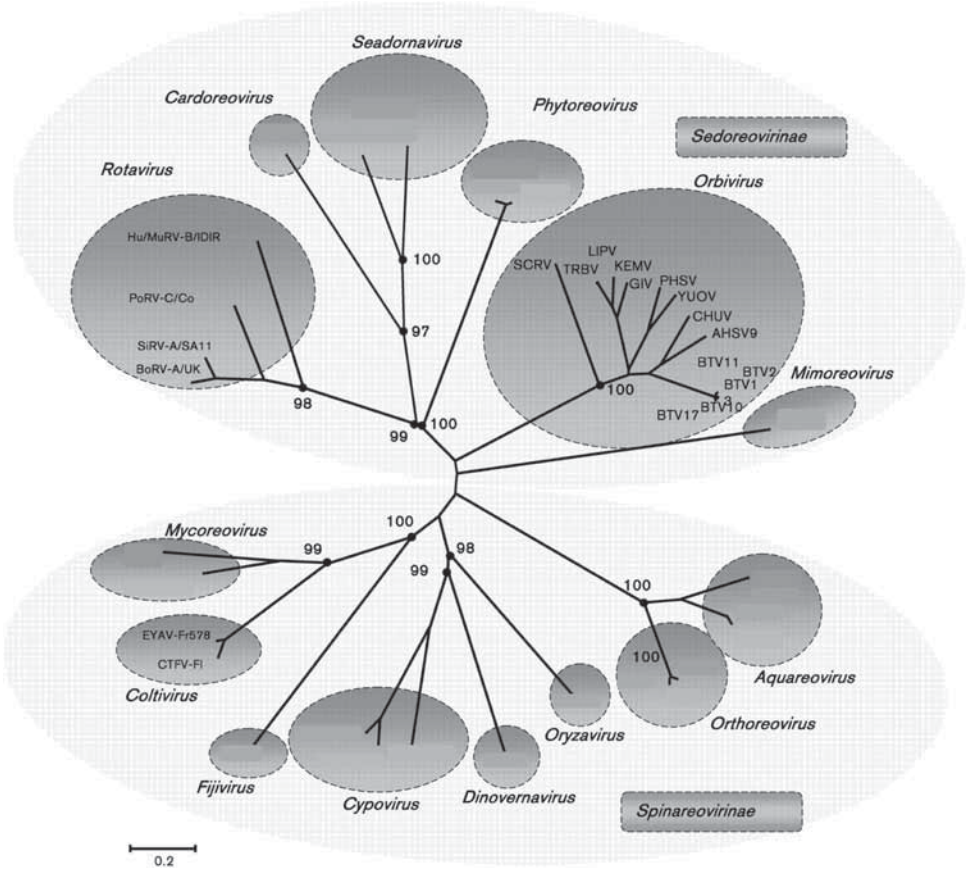

FIGURE 1 Relationship between members of Coltivirus, Seadornavirus, and Orbivirus with other Reoviridae based on phylogenetic analysis of the viral polymerase sequence (VP1). Each forms a distinct sequence cluster. Seadornaviruses appear most closely related to rotaviruses. KDV, Kadipiro virus; BAV, Banna virus; SCRV, St. Croix River virus; AHSV, African horse sickness virus; CHUV, Chuzan virus; BTV, Bluetongue virus; YUOV, Yunnan orbivirus; PHSV, Peruvian horse sickness virus; CTFV, Colorado tick fever virus; EYAV, Eyach virus; BoRV, Bovine rotavirus; SiRV, Simian rotavirus; PoRV, Porcine rotavirus; Hu/MuRV, Human/Murine rotavirus. Tree generated using MEGA 4 with a neighbor-joining method. Values at the nodes indicate bootstrap confidence. Bar, genetic distance or approximate number of substitutions per site. (Reprinted from reference 30 with permission of the publisher.)

to be determined. CTFV and EYAV have been fully sequenced. Nucleotide sequence homologies among CTFVs range from 90% to 100% for conserved segments such as genome segment 12, while homologies between CTFV and EYAV isolates range from 53% to 58% in this segment. These viruses constitute the *Coltivirus* genus (18–20, 23).

Previously grouped within the genus *Coltivirus*, the *Seadornavirus* genus comprises a distinct group of arthropod borne agents, principally found in East Asia (19, 20, 23, 29) (Fig. 1). The current taxonomy recognizes three distinct species:(i) the type-species, Banna virus (BAV); (ii) Kadipiro virus; and (iii) Liao Ning virus. The pathogenic potential of these viruses in humans remains to be determined, although associations of BAV infection with febrile illnesses and encephalitis are convincing. Seadornaviruses are serologically distinct from CTFV and EYAV, have a lower G+C content than coltiviruses (37% to 39% vs. 48% to 52%) and show genetic distances in the RNA-dependent RNA polymerase of more than 90% when compared to coltiviruses. Phylogenetic analysis based on sequences from the RNA-dependent RNA polymerase suggest that seadornaviruses are more closely related to rotaviruses than to other members of the *Reoviridae* (Fig. 1) (25, 30). Furthermore, structural studies demonstrate similarities between BAV proteins and those of rotavirus. The BAV outer capsid viral protein 9 (VP9) has homology to the rotavirus receptor binding protein VP8, and there are additional similarities between BAV VP10 and the VP5 domain of rotavirus VP4 (37).

At present, there are over 20 recognized orbiviral species comprising over 200 identified serotypes, as well as a number of as yet unclassified viruses (25, 29, 38). The best-studied members of the genus *Orbivirus* are a number of veterinary pathogens, including the type-species bluetongue virus and African horse sickness virus, but several other groups within the genus (including Changuinola, Kemerovo, Lebombo, and Orungo) appear capable of infecting humans with or without causing disease. In the southwestern United States, orbiviral infection with an agent(s) related to the Kemorovo-Lipovnik serogroup of the Great Island virus species or the Six Gun City virus (Chenuda virus species) is suspected to be the cause of an acute febrile illness characterized by myalgia, abdominal pain, vomiting, and pancytopenia (7, 29, 35). It seems quite likely that additional members of each genera of arthropod-borne reoviruses will be identified in the future (36).

Composition and Biology

CTFV particles consist of nonenveloped double-capsid structures with icosahedral symmetry and an outer capsid diameter of approximately 80 nm. While classified in the subfamily *spinareovirinae* based on sequence homology, CTFV spikes are not prominent and capsids appear smoother than is typically seen for either *seadornavirus* or *orbivirus* particles, with less prominent surface projections (23) (Fig. 2). BAV has a structure similar to that of rotaviruses, with protein fibers extending from the surface (37). Though CTFV does not acquire an envelope, electron microscopy studies frequently demonstrate association of viral particles with membrane (28,39–41).

The genome of all members of the *Reoviridae* consists of segmented dsRNA. Orbiviruses contain 10 segments, rotaviruses contain 11 segments, and coltiviruses and seadornaviruses contain 12 segments ranging in approximate molecular mass from 0.24 to 2.53 x 10^6 u (approximately 0.35 to 3.7 kbp). The 29 kB genome of CTFV is the longest of any *Reoviridae* characterized to date (18), while *Seadornavirus* genomes average 21 KB. Characteristic of the general genetic organization of the *Reoviridae*, each segment encodes a monocistronic message and gene product (22, 27, 28, 42–44). However, genome segment 9 of CTFV produces two different proteins through the use of a functionally "leaky" stop codon, allowing translation of both a truncated protein (VP9, a structural protein) and a longer, "read-through" protein (VP9') (45).

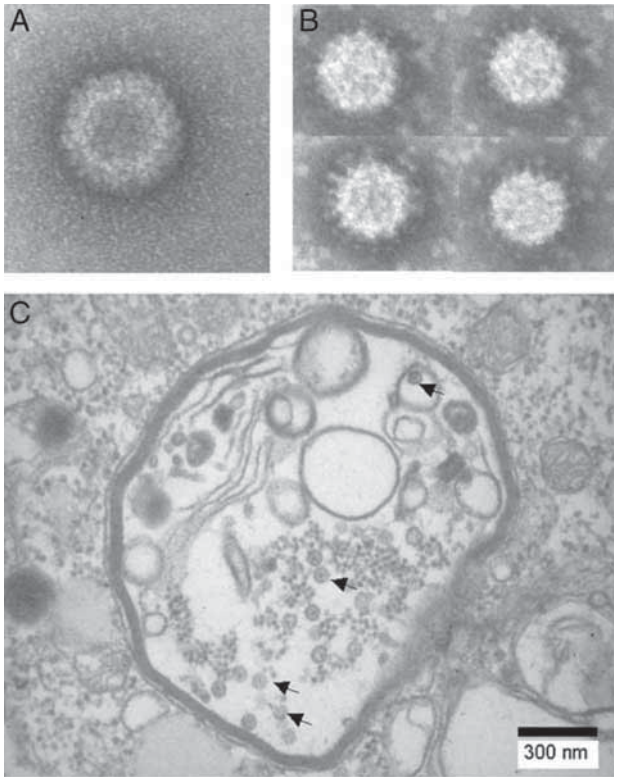

FIGURE 2 Negative contrast electron micrographs of (A) Colorado tick fever virus and (B) Banna virus (BAV). (C) Thin section of BAV-infected C6/36 cells showing viral particles (arrows) in vacuole-like structures. (Reprinted from reference 23 with permission of the publisher.)

Comparatively more is known about the gene products of orbiviruses and seadornaviruses than of CTFV. BAV (the prototype seadornavirus) has seven structural proteins. VP4 and VP9 form the outer capsid, while VP1, VP2, VP3, VP8, and VP10 form an inner core with a smoother surface (37). VP9 is involved in binding to the cell surface and may play a role in internalization (46). VP3 has guanyltransferase activity (47). VP1 of CTFV has sequence homology to and includes signature motifs characteristic of the RNA-dependent RNA polymerases of other reoviruses (18). Putative functions of other CTFV genes and their orbiviral homologs have been described previously (Table 1) (22).

The natural segmentation of the genome of *Reoviridae* allows reassortment, as has been shown by comparative studies of CTFV isolates acquired from the same location at different times. The adaptive advantage of this ability to reassort presumably outweighs the inherent cost of maintaining this segmented organization, both by permitting the generation of a larger number of potentially advantageous variants (positive selection) and by allowing salvage of portions of genomes that have suffered deleterious mutations because of the inherent infidelity of RNA genome replication (purifying selection) (48, 49).

Some genetic variability has also been demonstrated by differential polyacrylamide gel electrophoresis mobility of some of the RNA segments from different isolates obtained simultaneously from one location (50). Antigenic variation of CTFV is demonstrable by serum cross-neutralization studies (51). However, there is relative conservation of CTFV sequences based on RNA-RNA hybridization under conditions that allow hybridization with as low as 74% homology (52). A more complete understanding of the degree and role of genetic heterogeneity in the biology of CTFV awaits further characterization of individual gene products and comparative sequence data.

Studies on bluetongue virus have shown that the endocytic pathway is important to viral entry of orbiviruses and that completion of the replication cycle necessitates exposure to relatively low pH conditions (53). It is not known whether coltiviruses and seadornaviruses share similar entry requirements, but the observed similarities between the receptor binding proteins of BAV and bluetongue virus suggest that this may be the case (37).

Orbiviruses, coltiviruses, and seadornaviruses are all rendered noninfectious at a pH of 3. Unlike the orbiviruses, coltiviruses are relatively sensitive to treatment with deoxycholate, although with rare exception, members of each genus are relatively resistant to ether and other solvents (28, 54).

EPIDEMIOLOGY AND ECOLOGY

The geographic distribution of Colorado tick fever, which is defined by the distribution of the arthropod and vertebrate hosts for CTFV, consists of mountainous and highland areas at altitudes between 4,000 and 10,000 ft (1,000 to 2,500 m) in the western United States and southwestern Canada (Fig. 3) (7, 17). Because reporting of cases is not required, the several hundred cases reported annually likely underestimate the true annual incidence by a factor of 10 or more (1, 6, 16, 36). As an example, the declassification of Colorado tick fever as a reportable disease in Colorado in the early 2000s resulted in an apparent but misleading decline in disease incidence in that state. In contrast, active surveillance in Sublette County in Wyoming in 2009 resulted in the highest number of cases reported to the Centers for Disease Control from any one county or state since 1998 (1, 55).

TABLE 1 Coding organization of Coltivirus, Seadornavirus, and Orbivirus segments[a,b]

Coltivirus		Function	Seadornavirus		Orbivirus[c]	Function
CTFV	EYAV		BAV	KAV	BTV	
VP1	VP1	**RNA dep. RNA polymerase**	VP1	VP1	VP1	**RNA dep. RNA polymerase**
VP2	VP2	**Capping enzyme-methyltransferase**	VP3	VP3	VP4	**Capping enzyme-guanylyltransferase**
VP5	VP5	*Guanylyltransferase* (NS)	VP5	VP6		
VP3	VP3	RNA replication factors			VP3	Major subcore protein
VP6	VP7	**Nucleotide binding, NTPase**	VP6, VP2	VP5, VP2		
VP7	VP6	*RNA replication factor* (Protein kinase/NS)	VP7	VP7	NS1	Tubule protein
VP8	VP8	*Unknown* (Core)	VP8	VP9	VP7	Major core surface protein
VP9	VP9	*Structural and non-structural* (Core, stalk base)	VP10	VP10	VP2, VP5	Outer capsid protein
VP10	VP10	Kinase, helicase (dsRNA-binding)	VP12	VP8	VP6, NS4	Helicase, binds ssRNA, dsRNA
VP11	VP11	*Unknown* (Cell attachment)	VP9	VP11	NS2	Viral inclusion protein
VP12	VP12	*RNA replication Factor* (NS)	VP11	VP12	NS3	Virus budding viral release protein

[a]Adapted from references 7, 8, 12, 55, 95.
[b]Coltivirus functions shared by homologous segment of Seadornavirus and/or Orbivirus are listed in **bold**. When putative function of a segment from Coltivirus does not coincide with function in Seadornavirus, function in Coltivirus is *italicized* and function in Seadornavirus is in parentheses (). Functions of Coltivirus and Seadornavirus genetic segments are shown in column 3; functions of Orbivirus genetic segments are shown in column 7. Colorado tick fever virus (CTFV); Eyach virus (EYAV); Banna virus (BAV); Kadipiro virus (KAV); bluetongue virus (BTV); viral protein (VP); non-structural (NS).
[c]Although Orbivirus contain only 10 segments, segment 9 encodes both VP6 and NS4.

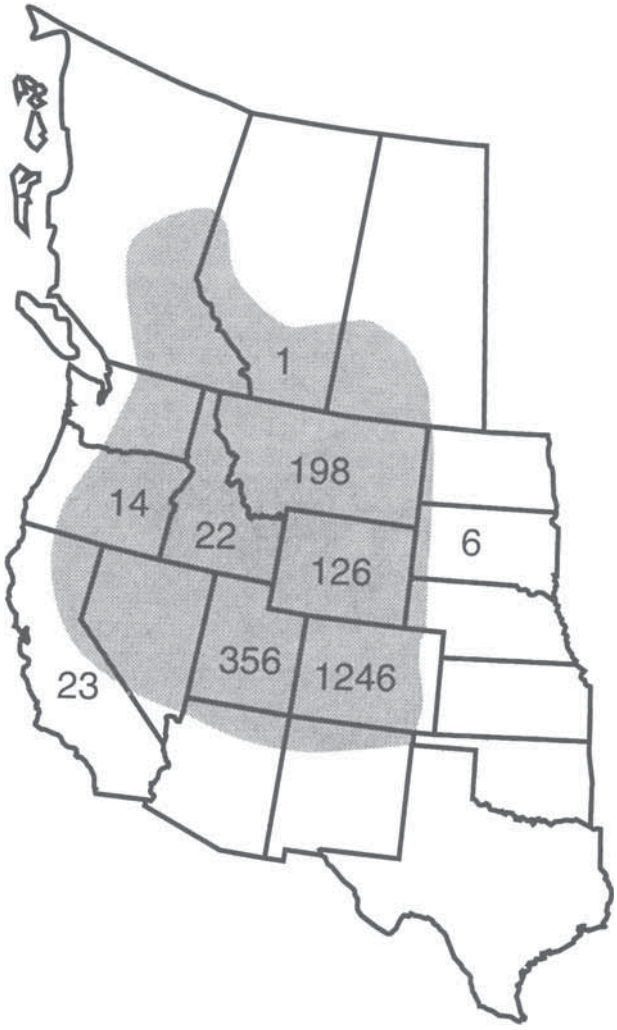

FIGURE 3 Distribution of *Dermacentor andersoni* ticks (shaded area) and number of cases of Colorado tick fever from 1990 to 1996. (Modified from reference 7 with permission of the publisher.)

Cases occur between March and October, with 90% of cases occurring between May and September (Fig. 4) (1, 17, 56). This seasonal distribution probably reflects the heightened numbers and activity of the arthropod vector *Dermacentor andersoni* (wood tick) and its natural vertebrate hosts (including the golden-mantled ground squirrel, Columbian ground squirrel, yellow pine chipmunk, and least chipmunk), as well as the greater exposure of human hosts participating in occupational and recreational activities during the summer months. Ticks are particularly numerous in grassy and low-brush areas, on south-facing slopes, and near streams (7, 17, 57).

In past studies, a preponderance of males between the ages of 15 and 40 comprised reported cases of Colorado tick fever. This does not stem from increased susceptibility but rather reflects the greater likelihood of exposure of this population to the vector carrying CTFV. There is no difference in likelihood of infection based on age or sex in populations when normalized for exposure (10, 58). More recent studies demonstrate a shift towards higher incidence rates among those 51 to 70 years of age. This possibly reflects greater case ascertainment among older patients, although a change in rates of exposure has not been excluded (1, 55).

Although CTFV has been isolated from several tick species, *D. andersoni* has been the only species demonstrated to transmit disease to humans (5). It has been postulated that *Dermacentor variabilis* may transmit CTFV or CTFV-Ca in regions of California that lie outside the area of distribution of *D. andersoni* (35). The life span of *D. andersoni* has been reported to be as long as 3 years, and once this species is infected, it remains so for life. Acquisition of infection can occur in the larval, nymphal, or adult stage with transstadial persistence, but transovarial transmission does not occur. Hence, passage of CTFV between generations of ticks requires an intermediate reservoir provided by the small mammalian hosts. These natural mammalian hosts develop subclinical infections followed by persistent viremia lasting weeks to months. Hibernating animals appear to sustain viremia for longer periods; this may be one mechanism that allows CTFV to survive the winter and to initiate a new cycle of infection when fed upon by larval and nymphal ticks in the spring and summer. The virus titer in an infected adult

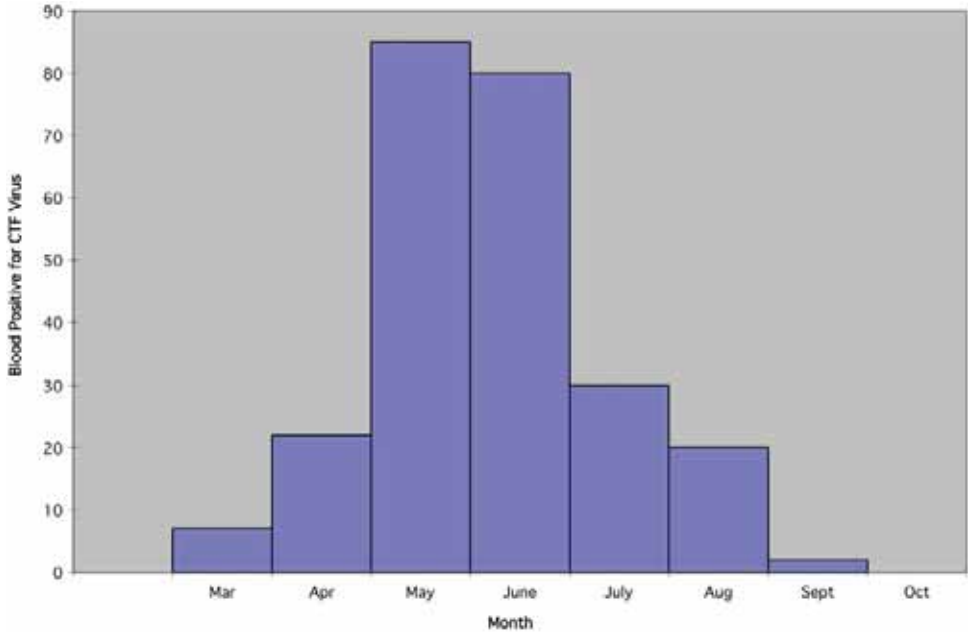

FIGURE 4 Frequency distribution by month of confirmed illness due to Colorado tick fever virus in Colorado during 1973 and 1974. (Modified from reference 56 with permission of the publisher.)

tick of 10^2 to 10^5 mouse 50% lethal doses/ml (homogenized tissue) can be maintained for up to a year. Transmission to humans or animals occurs by transfer of virus in saliva during feeding. Mature adult ticks prefer blood meals from large animals such as deer, elk, porcupines, and occasionally, humans (5, 7, 16, 36). Human-to-human transmission can occur during the viremic phase of illness, and there has been at least one documented case of transfusion-related Colorado tick fever (59).

PATHOGENESIS

Organ and Cellular Pathology

EM studies of cultured cells following infection reveal viral particles in association with granular matrices within the cytoplasm (40, 41). Filamentous arrays are also seen lying in parallel bundles, both in the cytoplasm and in the nucleus (nuclear filaments are not seen with other reovirus infections). In late stages of infection, the matrices become larger and viral particles become more numerous, but the viral particles are still contained within cells. There is no evidence for release of virus until cells undergo dissolution. EM of neurons infected *in vivo* fail to demonstrate a comparable cytopathic effect (23,39–41).

The tropism of CTFV for hematopoietic cells is demonstrated by the ability to detect viral antigen within erythrocytes by various methods (virus isolation, histochemical staining, fluorescent-antibody [FA] staining, and EM) long after the acute phase of infection (56, 60, 61). Since CTFV antigens are intracellular and not found on the erythrocyte surface, the virus may be shielded from immune clearance by neutralizing antibody throughout the natural life span of the erythrocyte. CTFV is able to infect and replicate in human hematopoietic progenitor cell lines, thus providing not only an explanation for the intraerythrocytic persistence but also a possible explanation for the frequently encountered leukopenia and thrombocytopenia (62). This pathology could be due to either a direct cytopathic effect on infected stem cells as they differentiate towards these cell lineages or a consequence of host immune clearance of those infected cells capable of displaying viral antigen. In patients infected with CTFV, mononuclear cells are also less able to produce colony-stimulating factors, and a circulating inhibitory factor (possibly lactoferrins or interferon) can be demonstrated in patient sera (63).

Only four deaths have been attributed to Colorado tick fever in the medical literature. Three were children and one was an 80-year-old man with underlying chronic lung and cardiac disease. Autopsies of two children, both of whom died from bleeding diatheses, reported purpura and cutaneous petechiae. Acute respiratory distress syndrome, encephalitis, and swollen endothelial cells were described in a 4-year-old boy. A 10-year-old girl had disseminated intravascular coagulation with focal necrosis involving the brain, liver, spleen, heart, and intestinal tract (4, 64). The adult patient presented with fever, diarrhea, and leukopenia and then developed disseminated intravascular coagulation and progressive respiratory failure. The patient developed immunoglobulin M (IgM) and immunoglobulin G (IgG) against CTFV; CTFV was recovered in tissue culture but equivocal IgM and positive IgG against hantavirus were also detected. Post-mortem findings in this lone published case of an adult death disclosed interstitial pneumonia (1). Studies on suckling mice and hamsters have disclosed histopathological findings similar to those described in these cases (65, 66).

Immune Response

High plasma levels of type I interferon (IFN) can be detected in a majority of Colorado tick fever patients during the first 10 days of infection. These levels appear to correlate with fever but not with other clinical parameters (67). Although the events responsible for upregulation of interferon expression in CTFV have not been described, they have been investigated for other *Reoviridae*. Infection of epithelial cells with bluetongue virus (BTV) upregulates expression of type I

IFN and related cytokines in a manner dependent on sensing and signaling by the cytosolic pathogen pattern recognition receptors retinoic acid induced gene-1 (RIG-I) and melanoma differentiation associated gene 5 (MDA-5) (68). In contrast, exposure of plasmacytoid dendritic cells results in MyD88-dependent but toll-like receptor (TLR) 7/8 independent IFN production (69). Overexpression of either RIG-I or MDA-5 in a cell line model impedes BTV infection (68). One third of patients develop detectable neutralizing antibody titers within 10 days of onset of symptoms. By 30 days, more than 90% are antibody positive, but many patients continue to have detectable viremia by either indirect FA assays or viral culture (presumably due to intraerythrocytic persistence) (56). Nearly 50% of patients are culture positive after 4 weeks and 5% to 17% are culture positive up to 12 weeks after onset of clinical symptoms. There is no apparent relationship between the persistence of viremia and the duration of symptoms. It is not known whether other immune mechanisms are involved in viral clearance.

Convalescence is accompanied by lasting immunity against reinfection. Only a single case has been reported of a patient experiencing either relapse or reinfection a year after the initial infection. Interestingly, the second episode was accompanied by loss of previously demonstrated neutralizing antibody titers (56). Experimental rechallenge of immune subjects with CTFV failed to produce clinical disease (with the exception of several hours of headache in some of the subjects) (10).

CLINICAL MANIFESTATIONS

Individuals with Colorado tick fever typically have a history of tick attachment or exposure (>90%) and residence or travel within endemic areas. Following an incubation period of 3 to 5 days (range 1 to 14 days), fever is noted along with malaise, headache, myalgias, and gastrointestinal upset (Table 2). In approximately half of all cases a characteristic "saddleback" fever pattern is seen. This consists of 2 to 3 days of fever, followed by an afebrile interval of up to several days, followed by return of fever for 2 to 3 days. Rarely, this can be followed by yet another febrile period. Thereafter, most patients recover without sequelae, although some have reported prolonged lassitude lasting weeks to months. Likely, mild or subclinical infection occurs but has been underrepresented in the literature (4, 5, 56).

TABLE 2 Symptom frequency of 320 patients suspected of having Colorado tick fever in Colorado for the years 1973 and 1974 (222 Confirmed positive and 98 negative)

Symptom	Positive for CTF (n=222) %	Negative for CTF (n=98) %
Fever	97	97
Headache	88	87
Myalgia	79	74
Lethargy	61	63
Abdominal pain*	21	33
Vomiting	24	24
Stiff neck	18	26
Sore throat†	19	34
Diarrhea	5	8
Skin rash	5	16
Bleeding	0	2
Petechiae	1	5
Vertigo	5	0

*Differences statistically significant ($p<0.05$).
†Differences statistically significant ($p<0.01$).
(Modified from reference 56 with permission of the publisher.)

Physical examination during the acute phase of disease can reveal altered sensorium, neck stiffness, photophobia, mild conjunctivitis, and occasionally lymphadenopathy and splenomegaly. Rash is seen in a minority of cases. When present, it appears as faint, fine macules or maculopapules on the trunk or, at times, the extremities (56). Hemorrhagic diatheses with attendant petechiae, particularly in those under age 10, have been attributed to thrombocytopenia and, in at least one case report, to frank disseminated intravascular coagulation (DIC) (4, 64). Central nervous system (CNS) involvement can range from self-limited meningitis to encephalitis with coma and death (6, 67). Other reported complications have included hepatitis, epididymo-orchitis, pericarditis, myocarditis, and pneumonitis (58, 59, 70–72). Rarely, deaths have been attributed to bleeding, pneumonitis, and CNS complications (1, 4, 6, 64).

There have been occasional reports of Colorado tick fever acquired by women during pregnancy, including one case of abortion 2 weeks after infection, one case of multiple congenital abnormalities in a mother infected in the first trimester (Colorado tick fever was thought not to be causal), and a case of apparent perinatal transmission with self-limited disease in the neonate (4, 73). No published reports describe the course of Colorado tick fever in human immunodeficiency virus-infected or other immunocompromised hosts, although one clinical review reports that immunocompromised individuals may be at risk for more severe disease (74).

Laboratory Findings

The most characteristic routine laboratory feature is a moderate leukopenia of 2,000 to 4,000 leukocytes/mm^3 or less, with a relative lymphocytosis (approximately 60% of patients). Other findings can include a "left shift" (at times with the appearance of metamyelocytes and even myelocytes) and toxic granulation. Thrombocytopenia can occur with or without DIC. Anemia (usually mild) may be present, but hemolysis is not a typical feature in the absence of DIC. There can be mild elevations of hepatic transaminases and creatine phosphokinase (5, 16, 36, 43, 56).

Mild to moderate cerebrospinal fluid (CSF) pleocytosis can occur, usually with a lymphocyte predominance (typical range, 0 to 500 mm^3), although there is a single case report of Colorado tick fever meningoencephalitis with a CSF leukocyte count of 1,578 and 89% neutrophils. CSF glucose has ranged from 40 mg/dl to normal and CSF protein has ranged from normal to 170 mg/dl in Colorado tick fever patients with meningoencephalitis (58).

Differential Diagnosis

Consideration of a diagnosis of Colorado tick fever must depend largely on epidemiological features. Because ticks can transmit a number of other infectious diseases, the differential diagnosis includes tularemia, RMSF, ehrlichiosis, Lyme disease, and tick-borne relapsing fever. Although the vectors for relapsing fever (*Ornithodoros sp.*), ehrlichiosis (*Dermacentor variabilis, Amblyomma sp., Ixodes sp.*) and Lyme

disease (*Ixodes sp.*) are different than the principal vector transmitting CTFV to humans, this distinction is often not made by patients or health care providers (7, 17, 75). Colorado tick fever must also be distinguished from a number of other viral diseases, in particular, those caused by the enteroviruses (because of the similar seasonal association) and in those cases with prominent CNS features, with West Nile virus. Distinguishing Colorado tick fever from RMSF can be difficult early in the course of disease. However, the typical improvement after 2 to 3 days, the possible appearance of the relapsing-fever pattern, the relative leukopenia, and the failure of the rash to evolve to a purpuric/petechial nature (as is seen with the vasculitic process of RMSF) would all point away from the diagnosis of RMSF (3, 5, 7, 16, 17, 36, 75).

The distributions of human monocytotropic ehrlichiosis (HME), caused by *Ehrlichia chaffeensis*, and human granulocytic ehrlichiosis (HGE or anaplasmosis), caused by *Anaplasma phagocytophila*, overlap only marginally with that of Colorado tick fever (76–78). *E. chaffeensis* is concentrated in the southeastern and south central United States, with sporadic cases reported from the Rocky Mountain and western states, consistent with the distribution of *Amblyomma americanum* (the Lone Star tick) in the southeast and south central United States and *Dermacentor variabilis* in the western United States (79, 80). HGE has been predominantly described in the upper Midwest (Minnesota and Wisconsin), Northeast (New York and Massachusetts) and, more recently, northern California, conforming to the geographic distribution of the *Ixodes* tick vectors (80–84). Nevertheless, it seems prudent that these two tick-borne diseases should remain in the differential diagnosis of suspected Colorado tick fever patients, especially those with severe disease. In particular, the cytopenias seen with HGE and severe cases of HME might be confused with that of Colorado tick fever.

LABORATORY DIAGNOSIS

Specific diagnosis of CTFV can be made by (i) viral isolation, (ii) demonstration of viral antigens, (iii) serology, or (iv) PCR-based detection of viral nucleic acids. Although CTFV can be cultured directly (in Vero or BHK-21 cells), the most sensitive method of isolation is intracerebral or intraperitoneal inoculation of blood clot suspensions or erythrocytes into suckling mice. The mice sicken and die 4 to 8 days after inoculation. Specific isolation is confirmed by FA staining of smears of mouse brain or blood or by cell culture. The blood clot intended for virus isolation should be stored refrigerated but not frozen. Typically, such procedures are available only through reference laboratories. When available, a source tick can be retained for species identification and virus isolation.

A more rapid, albeit less sensitive (60% to 70% compared with culture), approach to specific diagnosis is by direct immunofluorescence staining of blood smears for the presence of viral antigen (5, 7, 16, 17, 36, 43). Although diagnostic yield by either isolation or direct demonstration of antigen is greatest during the acute phase of disease, viremia is at times detectable well into the period of convalescence (56). Antigen is detectable in erythrocytes by direct FA assay in nearly 10% of patients as long as 20 weeks after onset of symptoms.

Paired acute- and convalescent-phase sera can be assayed for antibody titer in a variety of ways, including complement fixation (CF), indirect FA assay, enzyme-linked immunosorbent assay (ELISA), and an assay for neutralizing antibody measuring plaque reduction. CF antibodies may appear relatively late, whereas neutralizing antibodies are typically detectable from 14 to 21 days after onset of symptoms. IgM detectable by ELISA appears at about or slightly before neutralizing antibody but declines abruptly after 6 weeks (5, 16, 17, 36, 43, 85, 86). A recent ELISA for IgM using synthetic antigens and a separate Western blot analysis may be more sensitive than earlier ELISA systems for diagnosis but will require validation (87). Different ELISAs are also available for EYAV (using VP6) and BAV (using VP9) (88, 89).

Molecular diagnosis based on reverse transcription-PCR (RT-PCR) of RNA isolated from cell-free plasma or erythrocytes has been investigated for Colorado tick fever (87, 90). These methods appear to be comparable to or slightly more sensitive than viral isolation in the first week following infection but have the important advantage of providing rapid results and do not require the highly specialized reagents needed for virus isolation and identification. They should be superior to serologic assays for diagnosis in acute infection. However, these methods have only been applied to a limited number of viral isolates and retrospectively collected clinical samples and have not been prospectively tested except on experimentally infected animals (87). Furthermore, assays vary in sensitivity depending on virus strain, presumably due to sequence variation (90), although recent RT-PCR assays appear to be capable of detecting a single genome and are at least 10 times more sensitive than standard plaque assays (87, 91). Notably, some RT-PCR assays for CTFV do not detect the related CTFV-Ca, Salmon River tick fever virus, or EYAV (91). The utility and validity of these assays should increase when more complete sequence data become available from additional clinical viral isolates, permitting further assay refinement. Such approaches that permit early diagnosis are needed because the most important diagnostic and therapeutic decisions are faced during acute infection when clinical distinction from RMSF and other potentially more serious tick-borne diseases is most challenging. Microarrays that have been developed to simultaneously screen for multiple viral pathogens currently include probes for *Coltivirus* and *Seadornavirus* (92). Metagenomic approaches, for example, "deep sequencing," have been employed for pathogen diagnostics (93, 94) and discovery (95), including detecting other reoviruses (96). While there have been no reports of CTFV detection by these approaches to date, it seems likely in the future as assay methods improve and are more frequently used.

PREVENTION

Individuals should be educated and encouraged to take preventive measures when outdoors in endemic areas during the late spring and summer months. Wearing long-sleeved shirts and light-colored clothing, frequent self-inspection for ticks, and use of repellents such as DEET (*N*,*N*-diethyl-*m*-toluamide) or permethrin (only for treatment of clothing or bedding) should be advised (5, 7, 17, 43).

Patients recovering from Colorado tick fever should not donate blood for a period of at least 6 months because of the intraerythrocytic persistence of virus.

In the 1960s, a Colorado tick fever vaccine was developed using formalinized infected murine brain extracts (97). This vaccine elicits a neutralizing antibody response, but because of the modest morbidity of natural infection, the vaccine development program has since been abandoned.

TREATMENT

The typical, uncomplicated case of Colorado tick fever requires no specific therapy. Reassurance, analgesics, and

antipyretics can be given, with the caveat that aspirin and other nonsteroidal anti-inflammatory drugs with antiplatelet activities should be avoided (particularly in children) because of the occurrence of thrombocytopenia (5, 16, 17, 36, 43, 56).

In vitro activity against CTFV has been demonstrated for ribavirin, 3-deazaguanine, and to a lesser extent, 3-deazauridine (MICs of 3.2, 3.2, and 32 μg/ml, respectively, in MA-104 cells and MICs of 32, 3.2, and 10 to 100μg/ml, respectively, in Vero cells). In mice inoculated intracerebrally with CTFV, intracerebral or intraperitoneal ribavirin triacetate (but not ribavirin) reduced the mortality, while intracerebral 3-deazaguanine increased the mean survival time (98, 99). However, no experience with humans has been published. At present, no specific antiviral therapy exists for Colorado tick fever (100, 101).

SEADORNAVIRUSES

BAV (102, 103) was first isolated from the CSF and blood of patients with febrile syndromes and encephalitis (35, 104) as well as from mosquitoes (35, 102, 105) in southern and western China in 1987. Numerous additional isolates have since been reported from patients in China, including northern, more temperate regions, and Indonesia (106). Insect vectors for BAV include *Anopheles*, *Aedes*, and *Culex* mosquitoes. Viral sequences with similarity to BAV have also been found in cattle and pigs (107), suggesting a potential role for livestock as an animal reservoir. BAV is currently classified as a pathogen requiring biosafety level 3 laboratory containment and is the only species of the genus to be isolated from humans (23). BAV is endemic in areas of tropical and subtropical Asia that overlap with Japanese encephalitis virus and dengue virus, so it is likely that infection with BAV may be underestimated. Indeed, recent retrospective surveys of sera from Chinese patients with suspected Japanese encephalitis and other encephalitic syndromes suggest BAV infection in 8% to 12% (107). No specific treatment exists for BAV infection.

Kadipiro virus and Liao Ning virus are serologically distinct seadornaviruses that have also been obtained from mosquitoes in Indonesia and China (35, 102, 105). Sequence comparisons of the viral polymerase gene show homology ranging from 24% to 42%. However, these viruses have not been isolated from humans. Liao Ning virus is the only species of *Seadornavirus* that is able to replicate in a variety of mammalian cell lines and is also able to establish pathogenic infection in adult mice (108). A serologic assay for antibody to VP9 antigen (the outer coat protein) of BAV has been developed (89), as have RT-PCR assays for BAV and Kadipiro.

More recently, a virus detected in the intestinal contents of fresh water carp in Hungary by metagenomic analysis and confirmed by RT-PCR detection, Balaton virus, appears phylogenetically related to BAV, suggesting that the geographic distribution of *Seadornavirus* extends at least to Eastern Europe (96).

ORBIVIRUSES AND HUMAN DISEASE

The name *Orbivirus* derives from the appearance of virus particles as large, doughnut-shaped capsomeres by

TABLE 3 Orbiviruses that naturally infect humans[a]

Characteristic	Changuinola virus	Kemerovo virus[b]	Oklahoma tick fever virus[c]	Lebombo virus	Orungo virus
Geographic distribution	Panama	Russia and Eastern Europe	Oklahoma and Texas	Nigeria and South Africa	Western and central Africa
Insect vectors	Plebotamine flies	*Ixodes* ticks	Ticks (presumed)	*Aedes* and *Mansonia* mosquitoes	*Aedes*, *Anopheles*, and *Culex* mosquitoes
Reported clinical syndromes	Self-limited febrile illness in one adult	Fever, encephalitis, ? polyradiculitis	Fever, pancytopenia	Fever in one child	Fever, headache, myalgias, nausea, and vomiting (diarrhea, flaccid paralysis, seizures, normal routine CSF parameters in one child)
Specific diagnosis[d]	Rise in serologic titer; viral isolation in i.c.[e]-inoculated suckling mice, plaques in Vero and LLC-MK2 cell culture	Rise in serologic titer; viral isolation in i.c.-inoculated newborn mice, hamsters, chicken embryos; plaques in primary chicken embryo, Vero, and BHK-21 cell culture	Rise in serologic titer; viral isolation yet to be successful	Rise in serologic titer; viral isolation in i.c.-inoculated suckling mice, plaques in Vero and LLC-MK2 cell cultures	Rise in serologic titers; viral isolation in i.c.-inoculated suckling and weanling mice, plaques in Vero and BHK-21 cell cultures

[a]Data from references 110, 76, 55, 27, 30, 97, and 113.
[b]The Kemerovo strains include Kemerovo, Tribec, and Lipovnik viruses regarded as strains of the Great Island virus as well as at least 32 other strains that are not known to be pathogenic for humans.
[c]Serologically related to the Kemerovo strain of Great Island virus and the Six Gun City strain of Chenuda virus.
[d]Reagents to assay for various combinations of CF antibodies, neutralizing antibodies, IFA and direct nucleic acid testing are available at only a limited number of reference laboratories. Suspect cases can be referred to Division of Vector-Borne Infectious Diseases, CDC, Fort Collins, CO or to the U.S. Army Medical Research Institute for Infectious Diseases, Ft. Detrick,
[e]i.c., intracerebrally.

negative-contrast EM (orbi from Latin *orbis*, meaning "ring"). Although the most studied orbiviruses are agents of veterinary importance, a number of serogroups within the genus cause human disease (28, 43). They are predominantly transmitted by arthropod vectors (ticks, gnats, midges, and mosquitoes).

Changuinola virus was first isolated from a man in Panama experiencing a self-limited febrile illness. Serologic surveys in the area verify human exposure, but the frequency of clinical disease is unknown. The virus can be isolated from phlebotamine flies (43, 109). Four tick-borne viruses (Kemorovo, Tribec, Lipovnik, and Great Island) are currently classified as variants of the Great Island virus species (29, 30). Of these, Kemorovo has been implicated in febrile illness and CNS infection in Russia, and human seroreactivity to Kemorovo, Tribec, and Lipovnik viruses has been detected in parts of eastern Europe (33, 43, 110, 111). Serologic evidence of infection with a virus related to Lipovnik and Six Gun City viruses (a serotype of the Chenuda virus) was found in several patients from Texas and Oklahoma who were hospitalized with febrile illnesses associated with tick exposure and various degrees of transient leukopenia, thrombocytopenia and anemia. The etiologic agent(s) have not been successfully isolated (7, 112). The clinical syndrome has been given the name Oklahoma tick fever. Orungo virus has a wide geographic distribution in western and central Africa (43, 113). The principal vector is *Aedes* mosquitoes (though also isolated from *Anopheles* and *Culex*). The epidemiology of infection with Orungo virus therefore parallels that of yellow fever, and serologic surveys done during outbreaks of yellow fever demonstrate a high incidence of apparent coinfection with Orungo virus (114). In these studies, the contribution of Orungo virus to clinical illness was thought (understandably) to be small relative to that of yellow fever virus, but Orungo has been incriminated in febrile syndromes independent of yellow fever (115), and in at least one case it has been associated with CNS complications (43, 116). Lebombo virus has been isolated from a febrile Nigerian child and from *Aedes* and *Mansonia* mosquitoes in Nigeria and South Africa (43, 117). Lebombo and Orungo viruses are distinct serologically and by RNA-RNA hybridization and genetic reassortment studies (118).

Over several years in the 1980s, four laboratory workers in a South African veterinary vaccine packaging plant were apparently infected with neurotropic attenuated strains of African horse sickness virus (119, 120). Three of the four cases included frontotemporal encephalitis, and all four had evidence of uveochorioretinitis. The diagnosis was made based on specific serologic studies (CF, enzyme immunoassay, and neutralizing antibody) and the serologic exclusion of a battery of other viral and nonviral entities, including herpes simplex virus (though two of the four received 10-day courses of acyclovir) and Rift Valley fever virus. Several other laboratory workers demonstrated antibody to African horse sickness virus without overt clinical symptoms. The mode of acquisition was thought to be inhalation of lyophilized virus in laboratory accidents. Despite episodic outbreaks of African horse sickness virus and bluetongue virus around the world, natural human infection has not been reported.

Table 3 summarizes some of the clinical aspects of these naturally acquired human orbiviral diseases. As with coltiviruses and seadornaviruses, we are likely to encounter more orbiviruses in the future that have the capacity to cause human disease.

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Rotaviruses

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Acute infectious diarrhea is one of the two most frequent diseases of young children. Until the early 1970s, numerous unsuccessful attempts were made to grow viral agents responsible for acute infectious diarrhea of children. The etiologic agent of epizootic diarrhea of infant mice (EDIM) was identified by electron microscopy in 1963 (1). Nonetheless, it was only with the discovery of the virus responsible for calf scours (with this same approach) in 1969 by Mebus et al (2) and of the human Norwalk virus by Kapikian et al (3) in 1972 that the methodology for identification of the viruses responsible for severe diarrhea in children was established. Using electron microscopy, Bishop et al (4) identified the first human rotavirus in an intestinal biopsy from a child with diarrhea. At roughly the same time, other groups used immune electron microscopy to identify the enteric caliciviruses and astroviruses, viruses that were also difficult to grow *in vitro*, as additional causes of acute infectious diarrhea in children and adults. Shortly after the discovery of human rotaviruses, it was realized that the EDIM virus and the calf scours virus were morphologically and antigenically related, and all these strains were grouped in the genus rotavirus. In rapid order, rotaviruses were shown to be among the most important pathogens of acute diarrhea in the young of many animals, including humans.

Rotaviruses are responsible for a median of 39% (range 12% to 68%) of all hospitalizations in children under 5 years of age due to diarrhea worldwide, and it has been estimated that in 2008 (prior to the introduction of vaccines) they were responsible for the death of approximately 1,200 children daily (5) (Figure 1). In the Global Enteric Multicenter Study (GEMS study), conducted between 2007 and 2011 in countries with moderate-to-high mortality in children under age 5 in Africa and Asia, rotavirus was found to be the principal etiologic agent of moderate to severe diarrhea in children less than 59 months of age (6).

Rotaviruses do not account for a substantial number of deaths in developed countries, probably because of efficient and widespread access to rehydration and other supportive measures. Moreover, with the advent of rotavirus vaccines the impact of this pathogen has diminished in recent years in most developed countries. For example, in the United States detection of rotaviruses and costs for associated treatment have drastically diminished after vaccine introduction (7, 8). Although rotavirus was still identified in 12% of children with acute gastroenteritis in 2009 and 2010, during the same period norovirus replaced rotavirus as the leading cause of medically attended acute gastroenteritis in U.S. children (9). A similar situation may be occurring in some developing countries, as exemplified in Nicaragua where the epidemiology of diarrhea is changing (10).

VIROLOGY

Classification

Rotaviruses belong to the *Reoviridae* family of icosahedral, nonenveloped, segmented double-stranded (ds) RNA viruses. Rotaviruses are classified into five groups (A through E) depending on the presence of cross-reactive antigenic epitopes primarily located on the internal structural protein VP6. Among these, Group A rotaviruses (RVA) are the most frequent pathogens of humans, and groups D and E have been found only in nonhuman animals. Unless otherwise noted, this chapter will address only RVA. Group B rotaviruses (RVB) are sporadic pathogens of animals but have been implicated in several large outbreaks of adult diarrhea in China in the 1980s and less frequently in the 1990s (11). More recently, they have been identified in children and adults with diarrhea in India and Bangladesh (12, 13). Group C rotaviruses (RVC) are primarily veterinary pathogens but have been reported to be sporadically associated with diarrhea in children. The seroprevalence of these rotaviruses is relatively high in humans, especially those living in rural areas, suggesting transmission from animals to humans (14). However, in some countries such as India, prevalence of antibodies against RVC is the same in urban and rural populations (15). Despite occasional studies implicating the importance of RVB or RVC in humans, RVA continues to be by far the most frequently identified pathogen.

RVA have been serologically classified into subgroups depending on the presence of epitopes localized on VP6 (11). Four subgroup specificities have been defined: subgroup I (most animal and a few human strains), subgroup II (most human and few animal strains), subgroup I + II, and nonsubgroup I/nonsubgroup II, with only rare human strains belonging to these last two categories. The subgroup classification was useful in early epidemiological studies but is less commonly used today.

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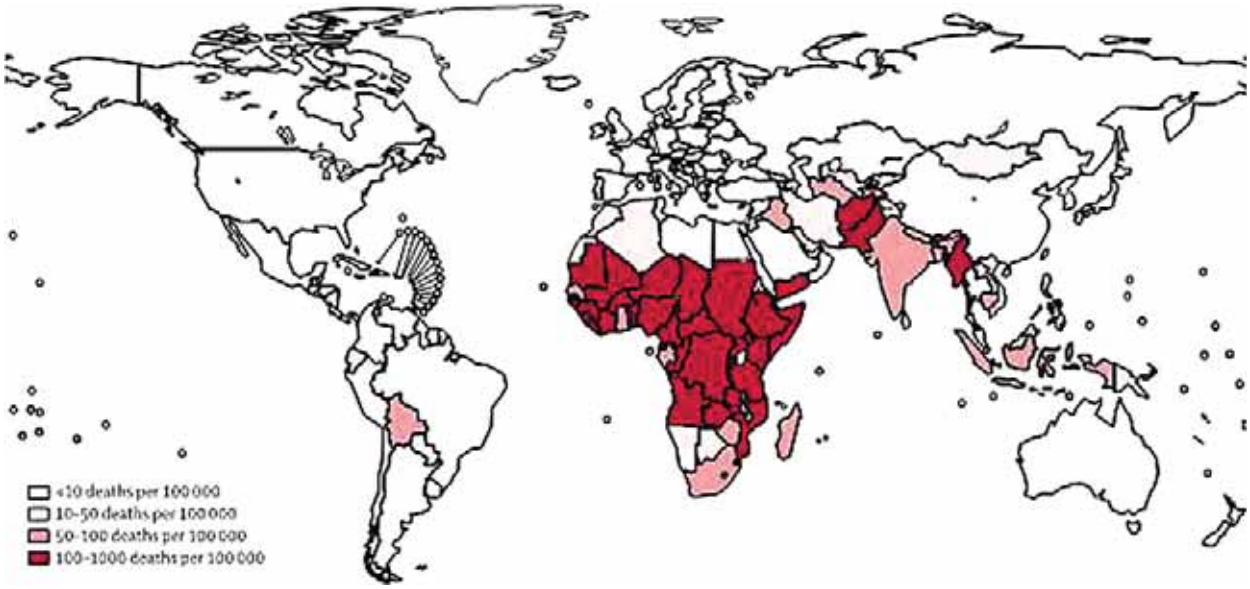

FIGURE 1 Map of the world showing rotavirus mortality in 2008, reproduced with permission from (5).

Antibodies that neutralize rotavirus *in vitro* are used to further classify the virus into serotypes. The two outer viral capsid proteins (VP7 and VP4) induce neutralizing antibodies and segregate independently, resulting in a binary serotyping system much like that of influenza. Antibodies against VP7 define the G (glycosylated protein) serotype and antibodies against VP4 the P (protease-sensitive protein) serotype. Historically, hyperimmune serum and later monoclonal antibodies (MAbs) against distinct rotavirus strains were used to classify viral serotypes.

The classification of G serotypes has been extensive and clear-cut because antibodies against VP7 generally predominate in hyperimmune sera, and serotype-specific MAbs to neutralizing epitopes on VP7 have been easy to isolate. The G protein has also been characterized based on its sequence analysis (genotype), and this classification correlates well with traditional serologic designations. In contrast, to obtain optimal P serotyping reagents it has been necessary to raise polyclonal antibodies against the different recombinant VP4 proteins (16) and these reagents have not been widely available. For this reason, many more distinct types of VP4 have been identified by comparison of gene sequence (genotype) than by serology. A significant but not absolute correlation exists between P serotypes and P genotypes. For example, the most common human P serotypes, 1A and 1B, correspond to genotypes [8] and [4], respectively, but genotypes [2] and [3] both can correspond to P serotype 5B (11). Worldwide, six G serotypes (G1- G4, G9, and G12) and three P (P[4], P[6] and P[8]) genotypes account for most human rotavirus infections (17). Human rotavirus strains belonging to G1, G3, G4, G9, and G12 serotypes are preferentially associated with P[8], while G2 serotype strains are most frequently associated with the P[4] genotype.

More recently, a complete genome classification system was developed for RVAs that assigns a specific genotype to each of the 11 rotavirus genome segments (18). The VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 genes of RVA strains are described using the abbreviations Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx (x = Arabic numbers starting from 1), respectively. The current guidelines recommend that nomenclature for individual strains include the following: rotavirus group/species of origin/country of identification/common name/year of identification/G and P-type (18). The prototype simian agent 11 (SA11) rotavirus strain is designated RVA/Simian-tc/ZAF/SA11-H96/1958/G3P5B[2] and the full genetic descriptor is indicated by G3-P[2]-I2-R2-C5-M5-A5-N5-T5-E2-H5 (18).

Composition of Virus

Virion Morphology

Rotaviruses, when studied with conventional electron microscopy (that does not permit clear identification of the VP4 spike protein), are 70-nm particles that have a multilayered icosahedral structure. *In vitro* treatment of a complete virus or triple-layered particles (TLP) with calcium chelating agents removes the outer viral capsid (VP4 and VP7), producing double-layered particles (DLP) composed of VP6 on the surface. Within the double-layered particles is a third layer (composed of VP1, VP2, and VP3), called the core, which contains the viral dsRNA genome. Pioneering structural studies of rotavirus using electron cryomicroscopy (19, 20) obtained detailed functional and structural information about this relatively large and structurally complex virus (Figure 2). More recent studies have elaborated an atomic model of the infectious particle (21, 22). The diameter of the viral particle, including the spikes, is 1,000 Å. Both outer and inner capsids are constructed with T = 13l (levo) icosahedral lattice symmetry. The 60 spike structures that protrude from the surface of the viral particle have been shown to be formed by trimers of VP4 that interact at their base with both the outer layer (VP7) and middle layer proteins (VP6) (23, 24). The structural changes of VP4 that probably occur during cell penetration recall those of enveloped virus fusion proteins (23). The localization of neutralizing epitopes on VP7 has also been identified (25) and insights into the mechanism of viral penetration obtained (26).

The particle has three types of aqueous channels that connect the central core containing the genome with the viral surface. Depending on their location relative to the icosahedral symmetry, these channels have been classified

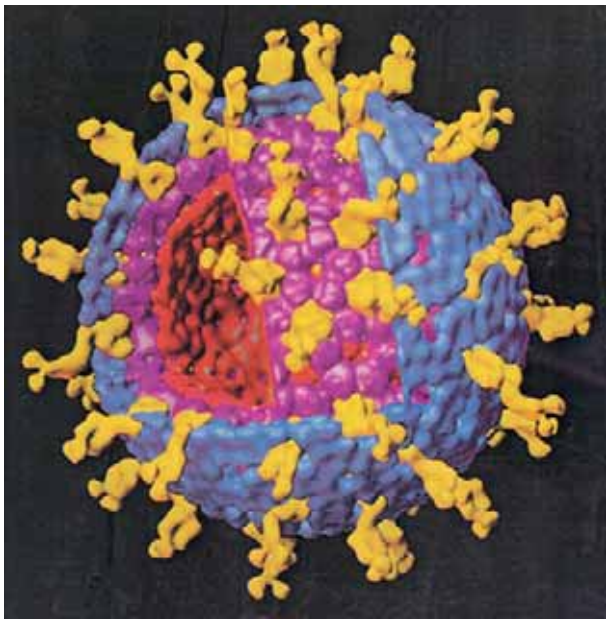

FIGURE 2 Computer image reconstruction of a rotavirus triple-layered particle based on cryoelectronmicroscopy. VP4, the spike protein, and VP7 compose the outer viral layer, in the cutaway, the middle layer is composed of VP6 and inside this layer, the core (that contains VP1 and VP3 and the viral RNA not seen) formed by VP2 can be seen. [Image courtesy of Mark Yeager and reproduced with permission from (19)].

into three types (I, II, and III) and are thought to be important in the entry of metabolites required for RNA transcription. Images of actively transcribing double-layered particles have been obtained (20) and the molecular localization of VP1 identified (27). The exit of nascent mRNA from the double-layered particles occurs via type I channels that do not differ in their structure from nontranscribing double-layered particles. Viral transcription may occur simultaneously from each of the 12 type I channels in the viral particle. Approximately one-fourth of the viral RNA is organized in an ordered dodecahedral structure localized near a VP1 (the viral polymerase), VP3 complex at the base of the type I channels (20). Further studies, in which actively transcribing particles have been incubated with MAbs that block transcription, have shown that the continuous translocation of nascent mRNA through the capsid is critical for efficient mRNA elongation and that blockage of translocation causes premature termination of transcription (28).

Viral Genome

Rotavirus contains 11 segments of dsRNA that range in size from 0.6 to 3.3 kilobase pairs with a total genomic size of approximately 18 kilobases. These segments are numbered according to size from largest (segment 1) to smallest (segment 11) (11). With the exceptions of segments 9 and 11 (which are biscistronic at least in some viral strains), each RNA segment contains a single open reading frame (ORF) with relatively short 5' and 3' terminal conserved noncoding regions. These conserved noncoding terminal sequences differ between RVA, RVB, and RVC and could be one of the factors that restrict reassortment between different groups of rotavirus. Positive strands of the dsRNA are capped at their 5' ends but do not posses a polyadenylation tract at their 3' end (11).

The genomes of rotaviruses are highly diverse. In decreasing order of relative importance, three primary sources for this diversity have been proposed: point mutations, reassortment, and rearrangement of the viral genome (11). *In vitro* studies have revealed a mutation rate of $<5\times10^{-5}$ mutations per nucleotide per viral replication cycle. This rate of mutation suggests that the average rotavirus genome differs from its parent genome by at least one mutation. Reassortment of gene segments also occurs at high frequency during mixed infections with two or more rotaviruses both *in vitro* and *in vivo*. In humans, the epidemiologic consequences of gene reassortment and the generation of serotypic diversity are much less dramatic than for influenza, although reassortment clearly occurs, especially in less developed countries (17).

Rearrangements (concatemerization, partial gene duplications, and deletions) of the viral genome segments have been observed, most frequently among rotaviruses recovered from chronically infected immunodeficient children (29). Rotaviruses with rearranged genome segments can also be generated *in vitro* following multiple passages at a high multiplicity of infection. Gene rearrangements generally involve the nonstructural proteins and VP6.

Viral Structural and Nonstructural Proteins

In addition to six structural proteins (designated as VP followed by a number), rotaviruses encode six nonstructural proteins (designated as NSP followed by a number). The gene coding assignments for these proteins were initially established with prototypic rotavirus strains that were easily cultured *in vitro*, like the simian table 3 SA11 strain (Table 1). A few hours after viral entry, perinuclear nonmembrane-bound, electron-dense cytoplasmic inclusions called viroplasms appear in infected cells, in which RNA replication, genome packaging, and the initial steps of assembly of double-layered particles take place.

VP1 binds the 3' end of the viral RNA, but its transcriptase activity is functional only in association with VP2 (22). The viral-like particles (VLPs) formed by VP1/2 are the minimal combination that supports RNA replication (11, 22). A conserved rotavirus specific motif present on the VP1 of RVA, RVB, and RVC is probably important for the structural or functional activity of the polymerase.

Baculovirus-expressed VP2 binds double-stranded RNA and DNA and assembles in the cytoplasm of infected cells in core-like particles 45 nanometers in diameter (11). The role of VP2 in the assembly of VP1 and VP3 and in replicase activity appears to be primarily structural and is linked to its ability to bind the mRNA template for minus-strand synthesis (11).

VP3 is found at the vertices of the inner core and it is a multifunctional capping enzyme because it has both guanylyltransferase and methyltransferase activities (30). Recently, VP3 was also found to contain a phosphodiesterase (PDE) motif and to have the capacity to inhibit RNase L-activation during the innate immune response (31).

VP4 trimers form spikes on the virion surface (24). VP4 has been shown to be the viral attachment protein both *in vivo* and *in vitro*, a determinant of viral growth *in vitro*, and a virulence factor *in vivo* (11, 32). Trypsin treatment of rotavirus cleaves VP4 into VP8* (amino terminal) and VP5* proteins and greatly enhances viral infectivity *in vitro* (11).

VP8* contains the most sequence variation in VP4 and determines the viral P genotype (11). Antibodies against VP8* neutralize the virus by inhibiting viral attachment. VP8* has been shown to be responsible for the ability of

TABLE 1 The dsRNA segments and encoded proteins of simian rotavirus SA11

dsRNA segment (Size bp)	Proteins (#: protein structure/ function)	Protein size: no. of aa (kDa)	Protein copies/ particle	Protein location	Functions
1 (3,302)	VP1 (Pol)	1,088 (125.005)	<25	inner capsid at 5 fold axis	RNA-dependent RNA polymerase, ssRNA binding, located at the 5-fold axis inside the inner capsid, part of minimal replication complex
2 (2,690)	VP2 (T1)	880 (102.431)	120	inner capsid shell	Inner capsid structural protein, sequence nonspecific RNA-binding activity, myristoylated, part of minimal replication complex
3 (2,591)	VP3 (Cap)	835 (98.120)	<25	inner capsid at 5 fold axis	Guanylyltransferase, methyltransferase, basic protein, part of virion transcription complex, phosphodiesterase (PDE), likely inhibits RNAse L activation
4 (2,362)	VP4	776 (86.782)	120	outer capsid spike	Trimers form outer capsid spike, P-type neutralization antigen, haemagglutinin, cell attachment protein, involved in cell tropism and virulence. Cleavage by trypsin into VP5* and VP8* enhances infectivity
	VP5*	529 *247–776 (60.000)			VP5* permeabilizes membranes
	VP8*	247 *1–247 (28.000)			VP8* hemagglutinin, highly variable sequence, and determines P genotypes
5 (1,611)	NSP1	495 (58.654)	0	nonstructural	Associates with cytoskeleton, extensive sequence diversity between strains, two conserved cysteine-rich zinc-finger motifs, RNA binding, has a role in suppressing the host IFN response, nonessential for *in vitro* replication in some strains
6 (1,356)	VP6 (T13)	397 (44.816)	780	middle capsid	Major virion protein, middle capsid structural protein, homotrimeric structure, subgroup antigen, myristoylated, hydrophobic region required for transcription
7 (1,049)	NSP3	315 (34.600)	0	nonstructural	Homodimer, specifically binds 3' end of rotavirus mRNA, binds eIF4G1, involved in cellular translational regulation
8 (1,059)	NSP2 (Vip)	317 (36.700)	0	nonstructural: in viroplasms	Nonspecific ssRNA-binding, accumulates in viroplasm, involved in viroplasm formation, NTPase and helicase activity, homomultimer (4–8 subunits), binds NSP5 and VP1, regulates NSP5 autophosphorylation
9 (1,062)	VP7	326 (37.368)	780	Virion surface glyco-protein	Outer capsid structural glycoprotein, G-type neutralization antigen, N-linked high mannose glycosylation and trimming, RER transmembrane protein, cleaved signal sequence
10 (751)	NSP4	175 (20.290)	0	nonstructural	Enterotoxin, receptor for budding of double-layered particle through ER membrane, N-linked high mannose glycosylation, uncleaved signal sequence, RER transmembrane glycoprotein, putative Ca^{++}/Sr^{++} binding site, secreted cleavage product. NSP4 is a pore-forming protein (viroporin) that elicits the release of endoplasmic reticulum (ER) lumenal calcium into the cytoplasm of the infected cell
11 (667)	NSP5	198 (21.725)	0	nonstructural: present in viral inclusion bodies	Interacts with NSP2 and NSP6, homomultimerizes, O-linked glycosylation, (hyper-) phosphorylated, autocatalytic kinase activity, binds ssRNA, component of viroplasm
	NSP6	92 (11.012)	0	nonstructural	Product of second out-of-frame ORF, interacts with NSP5, localizes to viroplasm, other functions unknown

#: Protein structure/function**:** RNA polymerase = (Pol); Inner virus structural protein with T = 1 symmetry = (T1); capping enzyme = (Cap); Inner virus structural protein with T = 13 symmetry = (T13); viral inclusion body or viroplasm matrix protein = (Vip). Other species of rotavirus within the genus may have proteins of different sizes.

*These proteins are produced by the VP4 gene.

Adapted from the table appearing in the previous edition of this chapter.

selected rotavirus strains to bind sialic acid and for the hemagglutinating activity present in many animal strains but absent in most human rotavirus strains. Structural analysis of the VP8* protein shows that it belongs to the galectin family of lectins expressed in enterocytes (33).

VP5* has been shown to function as an additional component of the rotavirus attachment complex (34). Also, VP5* is a specific membrane-permeabilizing protein which likely plays a role in the cellular entry of rotavirus (35).

VP6 is the major structural protein of the inner viral capsid (middle viral layer). VP6 is the most immunogenic viral protein and carries group- and subgroup-specific epitopes. The VP6 domains necessary for binding to VP2 are conserved in different rotavirus serogroups and are necessary for double-layered particle formation (11). Although not required for *in vitro* replicase activity, VP6 is required for the transcriptase activity, but its exact role in this function is unknown (36).

VP7 can be coded by gene segments 7, 8, or 9 depending on the viral strain (11). It is the major constituent of the outer rotavirus layer and the target of type-specific as well as heterotypic neutralizing antibodies. VP7 is a glycoprotein with three potential sites for N-glycosylation that are used variably, depending on the viral strain. Glycosylation of VP7 can influence its antigenicity. The retention of VP7 in the endoplasmic reticulum (ER) is mediated by a signal peptide sequence and other unique residues of the protein (11). Two neutralizing antibody epitope binding regions have been structurally defined for VP7 (25, 37), and antibodies recognizing them inhibit uncoating of the virion outer layer.

The amino acid sequence of NSP1, reported to bind zinc and RNA, is the most variable of all the rotavirus proteins. NSP1 has been implicated in host range restriction in the mouse model, although not in swine (11, 32), and appears to be dispensable for growth in some cell culture systems. Depending on the strain and host, NSP1 is used by the virus to evade the IFN response by promoting degradation of IRF3 or NFκB and inhibiting interferon-mediated STAT1 activation (38).

NSP2 interacts with NSP5, has helicase activity, and is present at high concentrations in the viroplasm, where viral replication takes place. NSP2 functions as an octamer with multiple enzymatic activities and it is considered the principal regulator of viroplasm formation (39).

NSP3 is a sequence-specific RNA binding protein that binds the nonpolyadenylated 3' end of the rotavirus mRNAs (40). NSP3 also interacts with the cellular translation initiation factor eIF4GI and competes with cellular poly(A) binding protein (PABP) (40). The competition between NSP3 and PABP for eIF4G has been postulated to be responsible for the shut-off of host cell translation seen during rotavirus infection (11). However, recent data suggest that rotaviruses reduce host protein translation by blocking the nucleocytoplasmic transport of polyadenylated mRNAs, in which NSP3 seems to play a role (41). Moreover, other recent data also suggest that NSP3 is a translational surrogate of the PABP-poly (A) complex and therefore cannot by itself be responsible for inhibiting the translation of host poly (A)-tailed mRNAs upon rotavirus infection (42).

The capacity of NSP4 to bind double-layered particles and insert them into the ER plays an important role in viral morphogenesis, and it is also a viroporin (11). NSP4 was the first putative viral enterotoxin described (43). A fragment of NSP4 is secreted from infected cells via a raft-dependent pathway (44). NSP4 is associated with virulence in mice (45), virulence of a porcine rotavirus, and host-range restriction of a human rotavirus (46). However, in humans and mice no clear association between virulence and specific NSP4 sequences has been identified (11).

NSP5 is a phosphoprotein with putative autocatalytic kinase activity localized to the viroplasm associated with NSP2, and it is a key protein during viral replication. NSP5 is present in infected cells as various isoforms, which vary according to different patterns of O-linked glycosylation (11).

NSP6 is the product of the second ORF of segment 11. It interacts with NSP5 and might have a regulatory role in the self-association of NSP5 and thus in viral replication (11).

Biology

Replication Strategy

Viral replication is understood mainly from *in vitro* studies (Figure 3). The process of viral attachment and entry is mediated by multiple interactions between rotavirus and the cell surface, and varies for different rotavirus strains (34, 47). Historically, RVA has been classified as sensitive to neuraminidase (NA) (most animal strains) or NA resistant (most human strains), depending on their susceptibility to the treatment of cells with NA and thus the presence or absence of sialic acid involvement in their attachment process (47, 48). However, NA-resistant rotavirus strains have been shown to bind to internal sialic acids that are not affected by NA (34). Moreover, P[8] and P[4] human rotavirus recognize H-type histo-blood group antigens (HBGA), whereas neonatal P[11] strains recognize the precursor of H-type 2 HBGA, referred to as type II glycans (47, 49, 50). Biochemical studies showing the involvement of N-glycoproteins, glycolipids, and cholesterol in rotavirus infection suggests that the integrins and other proteins involved in the attachment and entry process are likely to form part of lipid microdomains (rafts) in the cell membrane (34). However, inhibition of viral entry with agents specific for all the identified putative receptors/coreceptors decreases viral infectivity by less than 1 log, suggesting that more relevant entry factors are likely to be identified or that these known entry factors are redundant (34).

Host Range

Animal and human RVA can reassort *in vitro* and *in vivo*; for human RVA, multiple reassortment and interspecies transmission events contribute to their diversity (51). Nevertheless, there is a significant restriction of growth of rotavirus from one host species in another (host-range restriction); this restriction formed the basis for the development of Jennerian rotavirus vaccines (see the Vaccine section).

Growth in Cell Culture

Although initially growth of human rotavirus *in vitro* was very difficult, the discovery that trypsin treatment enhanced viral yield led to the cultivation of most isolates. Biochemical studies of rotavirus gene expression and function have been done both in the MA104 cell line (monkey kidney cells) and in polarized intestinal epithelial cell lines (52). The use of intestinal organoids (fragments of intestinal villi) has been recently proposed as a more physiological model to study rotavirus replication (53).

It is possible to construct infectious cDNA for many RNA viruses. However, transfection of purified rotavirus RNA or cDNA into permissive tissue culture cells, without helper viruses, has failed to yield infectious virus. Because of this, it has been impossible to efficiently use reverse genetics for rotaviruses, and researchers have been unable to manipulate the viral genome easily and to perform extensive functional studies of the viral genes. A reverse-genetics

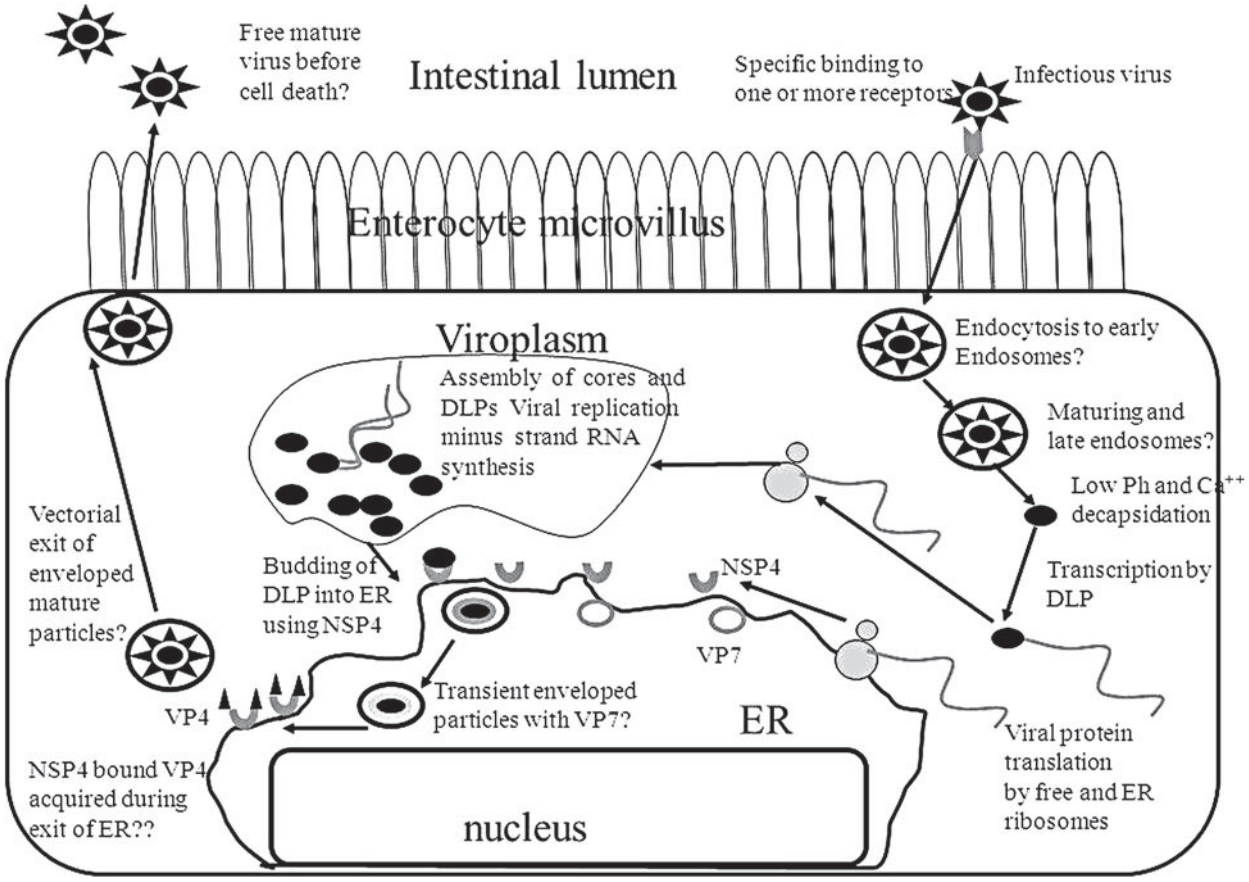

FIGURE 3 Schematic representation of the rotavirus replication cycle in intestinal polarized epithelial cells. The initial cell attachment step involves binding to cellular glycans and subsequent interactions with multiple receptors/coreceptors mediated by VP8* and VP5*, respectively (47). As a second step in entry, RVA have been proposed to interact with integrins α2β1, αVβ3, and αXβ2 and with the heat shock cognate protein hsc70 (34). Although not completely established, most studies suggest that rotaviruses enter cells by endocytosis (34) and then lose VP7 and VP4 (11). In the cytoplasm, double-layered particles gain transcriptase activity and begin to synthesize viral mRNAs. Viral proteins accumulate in the cytoplasm in an electron-dense region called the viroplasm, where the viral genome is replicated and the assembly of progeny double-layered particles takes place (22). An initial stage in viral assembly that is incompletely understood involves the assortment of single-stranded positive RNA strands corresponding to each one of the 11 gene segments (216). A viral intermediate particle that consists of VP1, VP2, VP3, and probably all the nonstructural proteins, except NSP4, is then formed (11). This complex acquires VP6 and during this process the nonstructural proteins are lost. Subsequently, the double-layered particles via VP6 interact with NSP4 that has been synthesized by ER ribosomes (217). This interaction leads to budding of the double-layered particles (DLP) into the ER lumen. In this organelle the particles acquire a transient lipid membrane. Subsequently the viral particles acquire VP7 and VP4 and lose the transient enveloping membrane. The exit of the mature triple-layered viral particles from the ER and the cell has been incompletely elucidated and may involve a raft-dependent pathway before cell lysis occurs (218, 219) or may be a postlysis event. The mechanism of cell death induced by rotavirus (before or after viral exit) is incompletely understood but is dependent on cell apoptosis *in vitro* and *in vivo* in mice (220, 221).

method to create rotaviruses with complementary DNA (cDNA)-derived reassorted genes was developed but, since this method relies on helper virus functions, it has proven relatively inefficient (54). At least two additional reverse genetic methods that also use helper viruses have been described (55, 56) that may prove more useful. As an alternative approach to study the function of individual viral genes, inhibition of specific RNAs with small interfering RNA (siRNA) (57) has proven quite useful.

EPIDEMIOLOGY

Distribution

Rotavirus infection occurs worldwide; most children develop antirotavirus antibodies by the age of 2 years. The incidence of rotavirus infection is comparable in developed and developing countries, implying that improvements in sanitation and hygiene will be ineffective for the control of this infection.

Incidence and Prevalence of Infection

The estimated incidence of rotavirus diarrhea varies depending on the method used for detection and case definition and on the epidemiologic settings under study. The incidence of rotavirus diarrhea defined by prospective studies in developing countries has varied between 0.07 and 0.8 episodes per child per year (6, 11). Compared with diarrhea caused by other microorganisms, the diarrhea produced by rotavirus infection is particularly severe and frequently associated with dehydration. Rotavirus infections account for a low percentage (8%) of all cases of community-acquired diarrhea,

28% of diarrhea associated with outpatient or clinic visits, and 39% of hospitalizations for diarrhea among young children (11).

The incidence of various rotavirus serotypes/genotypes varies by geographic location and time (17, 58). Of the 35 P and 26 G genotypes described to date, P genotypes [4], [6], [8], [9], [10], [11], [14], and [25] and G genotypes 1–4, 9, and 12 are epidemiologically important for humans (11, 58). A review of rotavirus genotyping studies from 1996 to 2007 characterized changes in RVA strains in the prevaccine era (58). Five genotypes (G1-G4 and G9) accounted for 88% of all strains, with temporal variation being evident. The prevalence of G1 strains declined from 2000 onward, while G3 strains reemerged, and G9 and G12 strains emerged during the same period. Rotavirus genotyping studies have been extensively performed in the postvaccine era (2007 to 2012) to determine if vaccine administration influenced the genotypes of circulating strains (17). Similar to the prevaccine era, the six strains most commonly reported in people were found in approximately 73% of the samples (G1P[8], 31.2%; G2P[4], 13.0%; G3P[8], 10.7%; G9P[8], 10.2%; G4P[8], 5.0%; G12P[8], 2.7%). Following prevaccine trends, temporal geographic variations in strain distribution have been observed in the postvaccine era. For example, in South America G2[P4] strains, and not G1[P8], are the predominant strain. In Africa, prevalence of viruses with genotypes P[6] and G8 was greater than 1%, including G2P[6] (8.2%), G1P[6] (6%), G3P[6] (5.5%), G12P[6] (3.5%), G8P[6] (1.7%), G8P[4] (1.8%), and G1P[4] (1.1%). Moreover, evidence for genetic interaction between vaccine and wild-type strains was observed, and transient predominance of heterotypic strains (G2P[4]) was observed mainly in countries using the Rotarix™ vaccine (the human monovalent vaccine referred to as RV1, see below), However, this finding requires further monitoring, and a clear effect of vaccine introduction on long-term rotavirus strain epidemiology has not been established.

Subclinical Infections

In a cohort of 200 Mexican children followed from birth to 2 years of age with weekly monitoring of rotavirus excretion in stool samples and regular serologic testing, approximately 50% of rotavirus infections were asymptomatic (59). The percentage of asymptomatic infections increased during the second, third, and subsequent infections, presumably as a result of the development of both homotypic and heterotypic immunity.

Epidemic Patterns and Seasonality

In the temperate zones of the world (mostly developed countries), rotaviral infection occurs primarily during epidemic peaks in the cooler months of the year (60). This pattern is not seen in countries within 10 degrees of the equator, where infection occurs in an endemic fashion throughout the year (60). No clear explanation is available for the rotavirus winter epidemic peaks; higher airborne transmission (60), higher stability of rotavirus with low relative humidity, or indoor crowding have all been proposed. However, clear association between lower relative humidity and development of rotavirus infections has not been found in all settings where this variable was analyzed (11).

Before the introduction of rotavirus vaccination, a yearly wave of rotaviral illness spread across the United States (61) and Europe (62) following peculiar spatiotemporal patterns. In the United States this pattern of spread correlated with variation in birth rates, thereby suggesting that the number of babies experiencing their first infection is one of the primary drivers of rotavirus epidemics (63). The high birth rates in developing countries may also influence the differential epidemiologic distribution of rotaviruses. The widespread use of rotavirus vaccine has greatly reduced or eliminated this spatiotemporal spread of rotavirus in the United States (64).

Age-Specific Attack Rates

The peak incidence of rotaviral illness in children in developing countries is between 6 to 11 months of age. In contrast, in developed countries like the United States and Canada, the highest rates occur during the second year of life. This difference between developed and developing countries has been proposed as due to the seasonal pattern of rotavirus infection in the developed countries; a child born just after the rotavirus season in a developed country would have almost a year before being exposed to the next rotavirus season, whereas a child born in a rotavirus-endemic country could become exposed throughout the year. Rotaviral infection is frequently asymptomatic in neonates (65–67), and neonatal strains isolated have been reported to have a particular P type and also specific differences in their VP7 and NSP4 genes when compared to pathogenic strains of similar serotypes (67). For this reason, it has been suggested that some strains found in newborns are naturally attenuated and could serve as vaccine candidates (65, 67). However, it is likely the newborn host rather than the viral strain is primarily responsible for the avirulent phenotype in newborns (66). The relative resistance to rotavirus illness in infants less than 3 months old may be a result of antibodies acquired by placental transfer from the mother (66) and possibly a difference in the glycans available for viral binding in the neonatal intestine (50).

Rotaviral disease of adults is seen occasionally in the elderly and in people who take care of sick children. In a prospective study of families, almost 50% of the caretakers of children with rotavirus diarrhea become infected with rotavirus, and 50% of these infections were accompanied by mild symptoms (68). In challenge studies of adults with virulent rotavirus, low levels of preexisting serum antibodies were associated with symptoms (69). Given the importance of rotavirus disease in the elderly, trials of pediatric vaccines are being implemented in this population (70).

Risk Factors and High-Risk Groups

Malnutrition, with its probable associated immunodeficiency, is a predisposing factor for severe life-threatening dehydration associated with rotavirus disease (71). In developed countries, low-birth-weight and premature infants appear to be at greater risk for hospitalization with rotavirus gastroenteritis (72). Interestingly, HIV has not proven to be a substantial risk factor for rotavirus illness (73), in contrast to severe combined immunodeficiency diseases (74). In the United States, although breast feeding was found to be protective, low-birth-weight infants, children in daycare, those covered by Medicaid or without insurance, and those having another child in the house had an increased risk of rotavirus hospitalization for rotavirus disease (75). African-American origin (76), maternal age, and maternal education less than high school may also increase the risk of hospitalization for rotavirus disease (75). On the other hand, in less developed countries breast feeding was not shown to protect against rotavirus illness (77). Thus, socioeconomic factors seem to be most important for increasing the risk of hospitalization with rotavirus gastroenteritis (75). Also,

three recent studies that implicate HBGA as RVA receptors have shown that the HBGA glycan can determine susceptibility to disease in a strain-specific manner (78–80); however, these results need to be confirmed.

Reinfections

In the cohort of Mexican children mentioned above, rotavirus reinfections were very common. Of the children studied, 96%, 69%, 42%, 22%, and 13% were reinfected 1, 2, 3, 4, and 5 times, respectively, during their first 2 years of life (59). The severity of disease rapidly decreased after the first infection and, remarkably, no child experienced moderate or severe disease after the second infection. In a more recent study in India, 56% of children were infected by 6 months of age (81). As in the Mexican study, protection against moderate or severe disease increased with subsequent infections but was only 79% effective after three infections.

Transmission

Rotaviruses are usually transmitted by the fecal-oral route, but some indirect evidence has suggested that they could also be transmitted by the respiratory droplet route (82). While nasal shedding of an attenuated human rotavirus strain in pigs has been reported, this phenomenon was less common when a virulent human rotavirus was examined in swine, suggesting that, if the pig model were relevant for study of human rotavirus transmission, then this mode of transmission might vary by viral strain (83). The origin of a rotavirus that infects a child who is not in contact with other children is unclear but may be due to subclinical infection in household adults (11). Although rotaviruses have been detected in both treated and untreated sewage water, waterborne outbreaks of rotavirus are probably rare because of the relative instability of rotavirus at high relative humidity (11). Foodborne outbreaks are also rare, although it has been reported that oyster and mussel samples can be heavily contaminated by rotavirus (84).

The efficient transmission of rotaviruses in the environment is assured, at least in part, because they are shed in the feces at a very high concentration (up to 10^{11} particles per gram) and are very resistant to degradation at ambient temperatures.

Institutions, such as daycare centers, with high concentrations of young children have an increased risk of developing rotaviral disease outbreaks. Moist surfaces including water fountains and water-play tables are common sources of rotavirus contamination in daycare centers (85). Rotavirus infection also occurs frequently in neonatal nurseries and, although most of these infections seem to be asymptomatic, some are not (86). Nosocomial rotavirus infections, especially in newborn nurseries, can be caused by a predominant rotavirus strain that differs from the strain currently circulating outside of the hospital (86, 87).

PATHOGENESIS IN HUMANS

Incubation period

The incubation period for rotavirus infection has been estimated to be less than 48 hours, based on animal studies and experimental infection of adult volunteers. This very short incubation period, which permits substantial viral replication prior to the amplification of a memory immune response, may be one of the factors that explains why multiple reinfections (generally mild or asymptomatic) occur with rotavirus (88).

Patterns of Virus Replication

The duration of viral excretion in infected children, determined both by ELISA and a sensitive PCR method, ranges from 4 to 57 days after onset of diarrhea (89). In about 43% of the children viral excretion stops in 10 days and within 20 days in 70% of the children. Extended excretion (mainly with the primary infecting rotavirus), but at generally substantially diminished levels, is detected for 25 to 57 days in the remaining 30% of the children.

An important study in cattle showed that rotaviruses penetrate but do not replicate in the intestinal M cells of Peyer's patches, an observation suggesting that rotaviruses can come in contact with the immune system by means of these cells (90). Although it was once thought that in immunocompetent children rotavirus infection was restricted to the mature enterocytes on the tips of the small intestinal villi (11), studies in both normal humans and animals clearly showed that this paradigm is incorrect. Most rotavirus infections are associated with antigenemia (91), RNAemia (92), and some level of viremia (93) in the initial phase of rotavirus-induced diarrhea in animals and children. In mice, the level and location of extraintestinal replication varies among rotavirus strains, and viral replication seems to occur in several leukocyte subsets (94). However, the clinical relevance of the findings of extraintestinal spread and replication is unclear, and in children and animals the greatest rotavirus replication occurs in the mature villus tip cells of the small bowel. Immunostaining with antibodies directed against nonstructural rotavirus proteins in autopsy samples from liver and kidney from severely immunodeficient children with chronic rotavirus infection suggests that viral extraintestinal replication occurs under these circumstances (74).

Pathology and Pathogenesis

The pathologic changes in the intestines of children infected with rotavirus include shortening and atrophy of the villi, mononuclear infiltration in the lamina propria, and distended cisternae of the ER (95). However, a direct relationship between the extent of histopathology and disease has not been demonstrated. For example, in a study of intestinal biopsies of children with rotavirus diarrhea, 95% of 40 patients did not have prominent histopathological changes despite symptoms (96). In the mouse model, rotavirus disease is associated with very modest histopathological findings, suggesting that the mechanism or mechanisms for the induction of rotavirus diarrhea could be similar in mice and humans. As noted above, the bulk of rotavirus infection appears to be restricted to the mature villus tip cells of the small bowel of all species (Figure 4), while low levels of viremia and extraintestinal replication probably take place in other organs. Using semiquantitative PCR, a correlation has been found between the levels of viral RNA in stool and the severity of diarrhea (97), suggesting that children with more severe diarrhea excrete more virus than children with less severe disease.

Study of the physiological basis of rotavirus-induced diarrhea in humans and in several animal models has yielded diverse and at times contradictory findings (95). In the pig model, rotavirus infection is associated with decreased intestinal lactase content, increased fecal lactose loss, and an increased fecal osmotic gap (95). These findings are consistent with the hypothesis that malabsorption of carbohydrates causes an osmotic diarrhea during rotaviral infection. In humans, a lactase deficiency has also been associated with

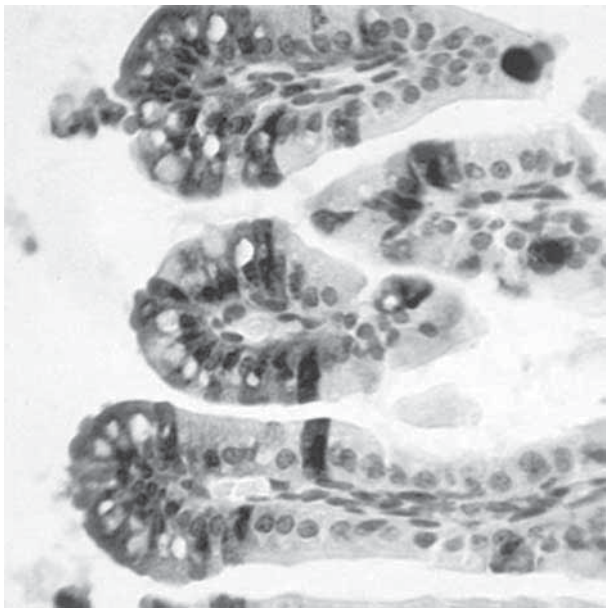

FIGURE 4 Cross-section of the small bowel villi from a rotavirus-infected mouse immunostained for rotavirus antigen. Note the restriction of growth of rotavirus to the mature villus tip cells of the small bowel.

rotavirus gastroenteritis (98). The most common explanation for the malabsorption and lactase deficiency associated with rotavirus diarrhea is the direct destruction of the enterocyte during viral replication. An alternative explanation that is more congruent with cases of rotavirus disease not associated with substantial pathologic findings is that rotavirus affects the turnover of disaccharidases in the microvilli (99). In support of this theory, it has been shown that rotavirus reduces sucrase-isomaltase expression and activity in human intestinal epithelial tumor cell lines by disrupting protein targeting and the organization of the microvillar cytoskeleton before apparent cell destruction occurs (100). In children with rotavirus diarrhea, some (98, 101) but not all (102) studies have shown increased mucosal permeability. A possible explanation for these *in vivo* findings has been suggested by *in vitro* experiments that show that rotavirus induces structural and functional alterations in tight junctions of polarized intestinal Caco-2 cell monolayers without alterations in cell and monolayer integrity (103). A recent study in mice did not find evidence for increased permeability at the onset of disease (104). The heterologous SA11 recombinant NSP4 and a synthetic peptide derived from it have been shown to induce an age-dependent secretory diarrhea in mice (43). In these studies, mouse pups born to dams immunized with the NSP4 derived peptide were partially protected from the diarrhea induced by a heterologous rotavirus strain. The homologous mouse NSP4 has also been shown to induce diarrhea in mice.

Because cystic fibrosis transmembrane conductance regulator (CFTR)-knockout mice, which do not respond to any known intestinal secretagogues, have homologous rotavirus-induced diarrhea, and NSP4 and the NSP4-derived peptide also induce diarrhea in these mice, NSP4 may mediate its effect through a novel secretory (non-CFTR-dependent) pathway or by another nonsecretory mechanism (11). Rotavirus also induces intestinal fluid and electrolyte secretion by activation of the enteric nervous system in the intestinal wall (105). In addition, rotavirus infection and NSP4 stimulate release of serotonin from intestinal sensory enterochromaffin cells *in vitro* and *ex vivo* (106). Mouse pups infected with rotavirus responded with central nervous system activation in brain structures associated with vomiting, providing an explanation for this symptom in rotavirus disease. Also in mice, rotavirus disease is associated with increased intestinal motility via activation of the myenteric nerve plexus (104). The fact that children with rotavirus gastroenteritis can be successfully treated with racecadotril, an enkephalinase inhibitor that can act on the enteric nervous system, also supports a role for this mechanism in rotavirus-induced diarrhea (107).

The three factors (malabsorption, NSP4, and enteric nervous system) proposed to explain rotavirus diarrhea could play different relative roles depending upon the animal species analyzed and, for a given species, could be operating together, with the relative importance of each mechanism varying depending upon the stage of infection. Determining the relative importance of each mechanism in human rotavirus-induced diarrhea might help in developing more effective vaccine strategies or new strategies to treat rotavirus diarrhea.

Immune Responses

As discussed above, although symptomatic reinfection with rotaviruses can occur throughout life, the severity and number of rotavirus infections diminishes with increasing age, and, in most settings examined to date, severe infections seem to be primarily limited to the first or second infection (88). This pattern of infection strongly suggests that a protective immune response against rotavirus develops after primary infection but that the generation of complete immunity requires multiple infections (108). Chronic rotavirus replication, prolonged symptoms, and extraintestinal infection in children with severe combined T and/or B immunodeficiencies (29, 74) also argue for an important role of the adaptive immune system in immunity to rotavirus. As is the case with severely immunocompromised children, some, but not all, strains of T- and B-cell immunodeficient mice become chronically infected with murine rotavirus (88).

Innate Immunity

Studies in mice have shown that rotaviruses are inactivated in the stomachs of adult but not newborn mice (109), which suggests that the development of gastric acid and pepsin secretion may be an important host defense factor against the virus. Lactadherin present in human milk specifically binds to rotavirus and inhibits its replication *in vitro* (110). Lactadherin levels in maternal milk are significantly higher in asymptomatic than symptomatic children with rotavirus infection, suggesting that this glycoprotein, rather than IgA, could be mediating an antiviral effect in milk.

The importance of innate immunity against rotaviruses has been indicated by experiments in which antibiotic treatment of mice reduces rotavirus infection in a toll-like receptor (TLR)-dependent fashion (111, 112). A potential mechanism of immune evasion is rotavirus stimulation of the secretion of TGF-β in polarized Caco-2 cells, and this effect has been shown to inhibit the capacity of dendritic cells to activate Th1 T cells (113, 114). Rotavirus has developed multiple mechanisms to evade interferon (IFN) innate immunity: NSP1, in particular, induces the proteosomal degradation of interferon regulatory factors (IRF) 3, 5, and/or 7, depending on the strain (115). In addition, NSP1 has recently been shown to inhibit the amplification phase of the

innate response by inhibiting STAT1 activation (38). Mice lacking the receptor for type III IFN, and to a lesser degree the receptor for type I IFN, have been reported to be more susceptible to murine rotavirus infection (116). On the other hand, STAT1 knockout mice (deficient in types I, II, and III interferon signaling) have been shown to be only marginally more permissive for homologous murine rotavirus replication (38). Hence, the relative roles of the IFNs in suppressing homologous rotavirus replication are not completely clear. However, the vomiting associated with rotavirus disease has been correlated with the increased levels of serum IFN-α found in children with acute rotavirus infections (117). Interestingly, interleukin (IL)-22 has been shown to act synergistically with type III IFN to restrict rotavirus replication (118). Finally, the combined effect of IL-22 and IL-18 is able to prevent and cure rotavirus infection in mice through a TLR5/NLRC4 mechanism (119).

Specific Humoral and Cell-Mediated Immune Responses

The immune response against rotavirus has been extensively studied in several animal models (88). Because rotavirus replication is primarily restricted to the enterocyte *in vivo*, the immune response against rotavirus originates in and exhibits its effector function directly at the intestinal mucosa. Intestinal T cells and neutralizing antibody responses are relatively weak in neonatal mice following homologous murine rotavirus infection (120, 121). Moreover, the presence of the integrin α4β7 (the intestinal homing receptor) has been shown to be required by B cells, but not $CD8^+$ T cells, for the migration of the lymphocytes to the intestine to provide immunity to rotavirus (88). The migration of the B cells to the intestine also depends on the presence of the CCR9 and CCR10 chemokine receptors (122). Although the studies in animal models have been enlightening, the immune response in humans may differ substantially. For example, while mice develop a primary lifelong protective intestinal IgA response against rotavirus, humans do not and can be reinfected multiple times.

A rotavirus-specific IgA coproantibody response occurs in 70% to 84% of children after a symptomatic infection (88). This IgA response has been reported to peak from 1 to 4 weeks after infection and then to decrease rapidly (108, 123). The relatively short duration of human intestinal antiviral IgA is probably one of the factors that contribute to multiple reinfections. During the acute phase of infection, IgM serum antibodies predominate and are subsequently replaced by IgG and, to a lesser extent, IgA (124). The IgG serum response is more durable than that of salivary or fecal IgA (125). During acute infection rotavirus-specific serum IgA has been shown to be directed principally against VP2 and VP6 and to broaden to include other structural and nonstructural proteins in the convalescent phase (126). Secondary infections will generally boost the fecal IgA response, and in many but not all children induce protective fecal antirotavirus IgA levels (108). Studies of human neutralizing antibody responses against rotavirus have shown that upon first exposures to rotavirus, children develop higher homotypic than heterotypic antibody levels (127). However, as the number of rotavirus infections increase, children develop more heterotypic antibodies.

To better understand rotavirus humoral immunity, specific B cells have been studied in both animals and children (128, 129). An important fraction of naïve human B cells bind rotavirus VP6 with low affinity (130), which highlights the importance of B cells as an innate component of immunity to rotaviruses. However, the Ig genes used by naive rotavirus-specific B cells are different than those expressed by memory B cells, suggesting that the latter do not primarily develop from the former (131, 132). Moreover, rotavirus-specific B cells are enriched in CD27+ memory B cells that express IgM (132, 133). Recently, using single-cell mass cytometry, intestinal and blood circulating rotavirus-specific B cells have been compared, and antibody secreting cells in the intestine and blood have been shown to be highly clonally related (134).

Based on ELISPOT and intracellular cytokine staining assays (135–138), healthy adults have circulating rotavirus-specific CD4 T cells that secrete IFN-γ or IL-2, whereas cells producing IL-4, IL-13, IL-10, or IL-17 were below detection limits. The frequencies of rotavirus-specific CD4 T cells producing IFN-γ in these subjects are comparable to those specific for other mucosal respiratory viruses (138). However, the majority of rotavirus-specific CD4 T cells were IFN-γ single producers, followed by a low percentage of double IFN-γ/IL-2 producers cells (138); they had the phenotype of terminally differentiated effector cells and were probably unable to provide long-term immunity (139). In agreement with their proposed intestinal origin, human rotavirus-specific CD4 T cells detected with a tetramer express both α4β7 and CCR9 (140).

Correlates of Immune Protection

Studies in animal models and humans have shown that local intestinal antibody is probably the primary protective effector mechanism against rotavirus (127). In mice, other mechanisms, such as $CD8^+$ T cells induced by natural infection, can also mediate modest levels of short-term protection (88). Protective humoral immunity in several, but not all, animal models, is associated with the presence of neutralizing antibodies directed at VP4 and/or VP7 (88). In mice, non-neutralizing IgA antibodies directed against VP6 are able to protect against viral infection (141), probably by mediating intracellular viral inactivation during transcytosis through the enterocyte (141, 142). In addition, suckling pups nursed by dams vaccinated with an NSP4-derived peptide can be partially protected from heterologous rotavirus diarrhea (43). However, in pigs (the only animal model in which protection from disease and not viral infection is measured) NSP4 antibodies do not correlate with protection (143). Thus, immunity may vary according to the vaccine, the animal species, the route of vaccination, and age of the vaccinees (121).

In studies performed in daycare centers and orphanages, in which antibodies to rotavirus have been measured very shortly before a rotavirus outbreak, intestinal and serum antibodies have correlated with protection against natural rotavirus reinfection (88). Rotavirus-specific antibodies (stool IgA in particular) have also been correlated with protection in some (108) but not in other studies involving naturally infected as well as vaccinated children (88). In general, serum antibody levels have been better correlated with protection following natural infection than following vaccination (144). At present, we do not have a precise and reliable marker of protection induced by vaccination (127). This deficiency has been an impediment to the development of new rotavirus vaccines because the only way of determining whether a vaccine is effective is in large-scale field efficacy trials. Both serum-neutralizing antibodies and serum and stool G-type specific antibodies have been shown to correlate with protection in studies carried out in an orphanage and daycare center as mentioned above (123).

Nonetheless, neutralizing antibodies have not been shown to correlate with protection in other settings (88). Likewise, in vaccine studies, some investigators (145) but not others have found a correlation between the presence of neutralizing antibodies and protection (88). The age at which children receive their first dose of the human RV1 seems critical in inducing neutralizing antibodies, and the frequency of children developing these antibodies is significantly below the level of protection induced by the vaccine (146). Thus, although neutralizing antibodies seem to play a role in protection, it is possible that antibodies against other proteins (VP6 and NSP4) or other mechanisms can also play a role in immunity. Recently, IgA antibodies against rotavirus have been shown to correlate with protection induced by RV1 (147). In addition, titers of rotavirus-specific IgA correlated with mortality in children under age 5 for both RV1 and RotaTeq™ (the bovine pentavalent rotavirus based vaccine referred to as RV5) (148). Polymeric antibodies (IgA or IgM) that have been secreted into the intestinal lumen can, by an unknown mechanism, be retrotranscytosed and reach the blood (88). In a small trial of a precursor to RV1, both titers of rotavirus-specific serum IgA and secretory antibodies correlated with protection, but this result needs to be confirmed (149).

The relative importance of maternal antibodies acquired transplacentally and from milk in protection against rotaviruses is unclear. In the pig model, antibodies acquired transplacentally (simulated by the passive transfer of serum from rotavirus-infected animals) or by colostrum could reduce the severity of primary rotavirus disease (150). These antibodies also inhibited the development of an effective mucosal immune response, and animals that received these antibodies were more susceptible to viral reinfection than the ones that did not receive the antibodies (150). It has been postulated that the relative resistance of newborns to rotavirus diarrhea is due to transplacentally acquired serum antibodies (66). Children breastfed with milk that contains antirotavirus neutralizing antibodies develop diarrhea to a similar extent as nonbreastfed children or those breastfed with milk lacking the antibodies (151). Breastfed children and nonbreastfed children seem to develop symptomatic or asymptomatic rotavirus infections to the same extent (110). The concentrations of lactadherin, but not rotavirus-specific secretory milk IgA, are higher in children with asymptomatic infection than in children with diarrhea. Thus, breastfeeding probably confers some limited protection against rotavirus but this effect is relatively minor compared to the effect breastfeeding has against several bacteria-induced diarrheas. Although some studies have shown slightly decreased vaccine "take" rates with breastfeeding (151), withholding breastfeeding during vaccination does not improve vaccine immunogenicity (152).

Correlates of Disease Resolution

The mechanisms that mediate disease resolution are multiple and probably redundant. In mice, CD8 T cells are probably the first immune mechanism that mediates rotavirus clearance (88), but in their absence antibody or other mechanisms can also perform this function.

Clinical Manifestations

Major Clinical Syndromes

The primary clinical syndrome caused by rotavirus infection is acute gastroenteritis. Typically, rotavirus-induced diarrhea is watery, lasts for approximately 5 days, is often preceded by the sudden onset of vomiting usually lasting 1 to 2 days, and is frequently accompanied by several days of fever (37.9°C or greater) and dehydration (153). Respiratory symptoms, although common in children with rotavirus infection, have not been specifically linked to this virus. Severe diarrhea in an age-susceptible child during the cool months of the year in a country with a temperate climate strongly suggests the diagnosis of rotavirus infection. Nonetheless, the clinical characteristics of rotavirus illness are not distinct enough to permit diagnosis based solely on physical examination and history, and laboratory confirmation of the diagnosis is necessary. However, laboratory diagnosis is seldom needed for therapeutic purposes in children with mild or moderate gastroenteritis.

Laboratory abnormalities in children with rotavirus infection are related to the extent of vomiting and dehydration (high urine specific gravity and electrolyte alterations) and should be investigated depending on the severity of these findings. The presence of reducing sugars in stool samples should alert the clinician to probable associated lactose intolerance (154). Occult blood and fecal leukocytes are found in small proportions, less than 16% and 39%, respectively, of rotavirus-infected children, but these findings are present at higher frequencies in children with bacterial infections and may suggest the differential diagnosis (155). Overtly bloody stools, prolonged diarrhea, leukocytosis, and a raised erythrocyte sedimentation rate also suggest a bacterial etiology (153). Children with rotavirus gastroenteritis often have elevated alanine aminotransferase (ALT) and aspartate aminotransferase (AST), suggesting that some hepatic involvement may occur during natural rotavirus infection (156, 157).

Rotavirus infection can cause severe and prolonged disease in children with severe combined primary immunodeficiency disease, some of whom shed virus chronically and develop disseminated infection (74). Acquired immunodeficiency also predisposes to severe rotavirus disease, and the virus can be a particular threat to severely immunosuppressed children with bone marrow and liver transplants (158). The role of rotavirus-induced disease in immunosuppressed adults with HIV and adult transplant patients is much less important (73, 159), although in some series death of patients with transplants has been associated with rotavirus infection (160).

Complications

Rotaviruses are not a major cause of prolonged diarrhea. However, diarrhea of rotavirus origin, especially in developing countries, can be the initiating factor for malnutrition with its accompanying immunodeficiency, which in turn makes children susceptible to other infectious diseases (161). Thus, the effects of rotavirus disease may not be limited to the morbidity and mortality associated *per se* with an episode of acute diarrhea. Some (but not all) studies have not found an association between natural rotavirus infection and intussusception (162–164). In mice, rotavirus enhances lipopolysaccharide (LPS)-induced intussusception (165). Moreover, natural rotavirus infection thickens the intestinal wall and enlarges mesenteric lymph nodes (166), suggesting a potential mechanism by which infection could promote intussusception. Studies aimed at clarifying this potential association are of great relevance, because RotaShield, the first licensed rotavirus vaccine, was withdrawn from the market because of its temporal association with very rare cases of intestinal intussusception. These cases mostly occurred in the first week after administration of the first vaccine dose (167). Although studies have failed to identify the reasons

RotaShield induced intussusception, it is important to note (see section on rotavirus vaccines) that this complication occurred mostly (80%) in children older than 3 months of age (168). A relationship between rotavirus infection and the development of diabetes has been proposed but not supported by experiments in mice or observations to date in children (169, 170). A possible association between RVC and the development of biliary atresia has been proposed by some (171) but not other studies (172). However, in rodents certain strains of heterologous simian RVA induce a biliary obstructive disease (173), which may be autoimmune in nature, since adoptive transfer of T cells from mice with rotavirus-induced biliary atresia into naive syngeneic recipient mice with severe combined immune deficiency resulted in bile-duct-specific inflammation in the absence of detectable virus. Also, some preliminary evidence suggests a possible association with celiac disease (174, 175). Seizures have been reported during rotavirus illness and in association with mild gastroenteritis caused by other viruses (176). The casual relation between rotavirus and seizures has been supported by the detection of rotavirus by PCR in some cerebrospinal fluid samples (176) and by the fact that rotavirus vaccination protects against childhood seizures (177). Electrolyte abnormalities and fever associated with infection could also explain some of the seizures, so a firm conclusion concerning the mechanism of seizures cannot be drawn at present. Many other rare complications have been associated with rotavirus infection, but due to the high frequency of rotavirus infections these associations probably occurred by chance.

Laboratory Diagnosis

Virus Isolation

Cell culture: At present cultivation of human RVA is a relatively straightforward task although rarely done in clinical laboratories. In contrast, very few RVB and RVC have been grown *in vitro*. Best results for growing human RVA are obtained using fecal samples (rectal swabs are less efficient) (11). With pretreatment of virus with trypsin (5 to 10 μg/ml) and subsequent incorporation of trypsin (0.5 to 1 μg/ml) in the medium of infected MA104 cells in roller tubes, 75% of human rotaviruses can be grown *in vitro* (11). Primary simian kidney cells (178) and intestinally derived cell lines such as the human colon adenocarcinoma cell line CaCo-2 (179) are probably superior to MA104 cells for primary isolation of rotavirus.

Antigen Detection

The first method available for making the diagnosis of rotavirus infection was electron microscopy. Although this method has generally been replaced by the more readily available and sensitive solid phase immunoassays, it can still be of use, for example, for detecting nongroup A rotavirus and mixed infections with other enteric viruses. Of the commercially available latex agglutination tests and ELISA kits for the detection of rotavirus in stool samples, the latter are more sensitive and are probably the most commonly used assay for making the diagnosis of rotavirus infection (180). The commercial ELISA kits available can have a sensitivity and specificity of up to 98% and 100%, respectively. Among the ELISA tests, those that employ specimen-specific negative controls (preimmune serums as capture antibodies) are superior in minimizing false-positive reactions (180). ELISA kits that use G- and P-serotype-specific MAbs are also available to type the most frequent rotavirus strains that infect humans.

Nucleic Acid Detection

Because in the early rotavirus studies it was impossible to culture the different viral isolates, the characteristic migration patterns of RNA during polyacrylamide gel electrophoresis were an important laboratory and epidemiological tool. This method of differentiating rotavirus strains is called electropherotyping. Although this method is still useful to differentiate RVA from rotavirus of other groups, among group A strains the different electropherotypes do not correspond to specific serotypes and are of limited value (11).

PCR amplification of rotavirus nucleic acid from stool specimens is the most sensitive way to detect RVA, RVB, and RVC (181–183). With this method, in a case control study up to 29% of asymptomatic children less than 1 year of age were positive for rotavirus (183). Although it is not clear that the RNA detected reflects active viral replication, symptomatic children generally shed much more viral RNA than asymptomatic children. A correlation has been observed between the severity of rotavirus diarrhea and the quantity of RNA shed (97). G (184) and P genotyping by PCR (185) is more sensitive than serotyping using ELISA with specific serotype-specific MAbs.

Serologic Assays

Detection of rotavirus-specific serum and stool antibodies is generally performed by ELISA and typically is restricted to epidemiological and vaccine studies. The most sensitive and rapid immunologic test to diagnose primary infection seems to be the detection of virus specific IgM in serum (124). A four-fold increase in convalescent as compared to acute serum titers of IgA and IgG can also be used to diagnose primary infections. An increase in IgA titer in convalescent stool samples is a more sensitive marker of rotavirus reinfection than is seroconversion (125). However, measurement of these antibodies in breastfed children is complicated by the fluctuation of these antibodies as a result of the presence of maternal milk IgA. Measurement of rotavirus-neutralizing antibodies is commonly performed by plaque reduction or focus reduction assay (146). Serotype-specific responses to defined epitopes of VP7 can be measured by an immunoassay that uses neutralizing MAbs to compete with the test serum as an alternative to traditional neutralization assays (186).

Prevention

General

Strict isolation of rotavirus-infected hospitalized patients is unnecessary, since the risk of nosocomial rotavirus infection is not enhanced by room contact with a rotavirus-infected patient or by the sharing of attending personnel. Precautions should be exercised in disinfection of surfaces thought to be contaminated with rotavirus, since these viruses have been shown to be highly resistant to many commonly used disinfectants. A spray composed of 0.1% o-phenyl phenol and 79% ethanol is highly effective in decontaminating surfaces with rotavirus (187). These measures, combined with careful hand washing, will limit the spread of infection in hospitals or other institutional settings. Nonetheless, the fact that the incidence of rotavirus infection is similar in developed and less developed countries suggests that these measures will not replace the need for an effective vaccine.

Passive Immunoprophylaxis

The feasibility of passively protecting newborns by immunizing mothers with rotavirus has been demonstrated in animal models and human trials in which oral vaccination of

mothers increased virus-specific antibodies in breast milk (188); however, the protective efficiency of this strategy in humans has not been evaluated.

Active Immunization

Because animal and human rotaviruses share antigens capable of inducing protective immunity (189), Jennerian vaccines (vaccination of humans with a naturally attenuated animal virus) have been the most extensively tested for rotavirus. A quadrivalent vaccine with the four most common human G serotypes was made by combining a Rhesus rotavirus (serotype 3) and three mono reassortants of this virus that possessed the gene for VP7 of human rotavirus serotypes 1, 2, and 4. The quadrivalent Rhesus vaccine (RotaShield, Wyeth/Lederle) was licensed for use in the United States but then withdrawn from the market because of its association with intussusception (167). However, the impact of this vaccine on the total attributable risk of intussusception was relatively small (estimated at 1/10,000) and age dependent (190).

Two live-attenuated rotavirus vaccines are currently licensed for use in many countries worldwide and are recommended by the WHO (191). The vaccine produced by Merck (RotaTeq^{TM}, or RV5) contains five monoreassortants of a bovine virus with G1, G2, G3, G4, and P1A[8] human rotavirus genes given in a three-dose schedule (192). The vaccine produced by GlaxoSmithKline (GSK, Rotarix^{TM}, or RV1) is an attenuated human G1P1A[8] virus given in a two-dose schedule (193). In trials that involved over 60,000 infants, both of these vaccines were shown to be safe and to provide protection against any and severe rotavirus diarrhea of over 70% and 98%, respectively. Importantly, both vaccines reduced the rates of all gastroenteritis related hospitalizations of any cause by over 40% (127). However, in developing countries in Africa and Asia these vaccines are less effective (194–196). The recommendation of the Advisory Committee on Immunization Practices (ACIP), which has been endorsed by the American Academy of Pediatrics, is that three doses of RV5 be given at 2, 4, and 6 months of age with an interval of at least 4 weeks between doses. Two doses of RV1 are recommended to be given at 2 and 4 months of age (197). Both vaccines are contraindicated for 1) infants with a history of severe allergic reaction (e.g., anaphylaxis) after a previous dose of rotavirus vaccine or exposure to a vaccine component, 2) infants diagnosed with severe combined immunodeficiency (SCID), and 3) infants with a history of intussusception (198). Both RV1 and RV5 can be coadministered with other childhood vaccines. Initially, for both vaccines the maximum age for dose 1 was 14 weeks and 6 days of age and for the last dose was 8 months of age, but recently WHO removed the age restriction for rotavirus vaccination after considering that benefits outnumber the potential excess vaccine-associated intussusception deaths (191). Nonetheless, early immunization is still favored (as soon as possible after 6 weeks of age), but each country establishes its own policy concerning age restrictions for vaccine administration, and rotavirus vaccination of children older than 24 months of age is not recommended.

Rotavirus vaccines have had a rapid impact on diarrhea induced by rotavirus and all other causes (199). After introduction of vaccines in the United States, rotavirus and all-causes diarrhea hospitalizations have declined 60% to 83% and 29% to 50%, respectively, in children less than 5 years of age (199). Indirect protection of older unvaccinated children and young adults has been detected in several developed countries, and more importantly, Mexico, Brazil, and Panama also detected declines in diarrhea mortality following vaccine introduction (199). Postlicensure monitoring of increased risk of intussusception has been conducted in some countries with similar results: e.g., 1 to 5 excess cases per 100,000 vaccinated children in U.S. studies. Also, both DNA from porcine circovirus types 1 and 2 were detected in rotavirus vaccines; however, these viruses are not known to cause human infection or disease and are very common in pigs. Again, WHO and regulatory authorities considered that benefits of vaccination greatly exceed these low risks and reaffirmed the global recommendation for use of rotavirus vaccines (199).

In addition to the newly licensed vaccines, several other live oral vaccines are being evaluated or in use (127): The Lanzhou lamb rotavirus strain, which was developed in China and used in the United States since 2000, induces only partial protection when given to children between 9 and 35 months old (200). A bovine reassortment vaccine developed in the United States by the National Institutes of Health has been shown to provide high levels of protection, and, in a new approach to vaccine development, this vaccine was licensed to seven companies in three developing countries, where it is currently under development and evaluation (201). This strategy may be be useful to lower the price of rotavirus vaccines. Based on the observation that neonatal rotavirus infection protects against severe rotavirus gastroenteritis later in life (65), vaccines derived from neonatal rotavirus strains (the Indian 116E and the Australian RV3 P[6]G3) are also under evaluation (202, 203). The 116 E candidate was recently shown to be safe and effective in a large Phase 3 trial in India (203) and is now licensed and being marketed in India under the trade name of Rotavac®. As a strategy to lower the risk of intussusception, some investigators are proposing neonatal administration of live oral vaccines, since at this young age intussusception is very rare (204). Also, to try to minimize this complication, strategies for future rotavirus vaccines include inactivated and several nonlive vaccines (e.g., recombinant viral antigens [VP6]), rotavirus-virus-like particles, DNA vaccination with selected rotavirus genes and vaccination with synthetic peptides (127). It is unknown if current rotavirus vaccines directed at RVA are cross-reactive with RVB or RVC, and vaccines against the latter agents have not been tested in humans.

Treatment

Since rotavirus disease spontaneously resolves in a few days to 1 or 2 weeks without treatment, therapy is aimed at preventing dehydration, which is the main serious complication (205). The standard hydration solution in use was derived from the formula initially used to treat secretory cholera diarrhea and thus has a high sodium concentration and an osmolarity of 331 mmol/L. Rehydration formulas with reduced osmolarity (224 mmol/L) have been suggested to be superior for treatment of children with non-cholera-induced diarrhea, especially the subset of patients with the most severe persistent disease (191, 205).

Several studies have indicated that passive oral immunotherapy (antirotavirus immunoglobulin from bovine colostrum, for example) can shorten the duration of rotavirus infection in animals and humans. The rationale for this type of treatment has been studied while treating immune-compromised children with chronic rotavirus diarrhea (206). More recently, a llama-derived, heavy-chain antibody fragment specific for rotavirus reduced stool output in male infants with severe rotavirus diarrhea (207). It is unclear,

however, whether these strategies will be economically feasible or logistically practical. In animal (208, 209) models and children (210), administration of some strains of *Lactobacilli* (the bacteria present in yogurt) can stimulate a stronger immune response to rotavirus and shorten the duration of diarrhea. These bacteria are considered to be safe, are currently recommended for use in Europe (211), and a clinical report by members of the American Academy of Pediatrics finds that there is evidence to support their use for the treatment of gastroenteritis, although more studies are necessary (212). Because of the associated side effects, such as ileus, the use of opiates and atropine are contraindicated for treatment of children with diarrhea. Several other compounds, such as racecadotril (an enkephalinase inhibitor with antisecretory and antidiarrheal actions) (107) and nitazoxanide (213) have been shown to be safe and effective in rotavirus-induced diarrhea but have been tested only in limited clinical studies (205). Zinc supplementation is effective in preventing and treating diarrhea in children in developing countries, but its use in developed countries needs further evaluation (191, 205).

Early refeeding after rehydration of children with diarrhea is recommended (214). A meta-analysis found that this practice does not prolong diarrhea and it may reduce the duration of diarrhea by approximately 0.5 day (214). Recommended foods include complex carbohydrates (rice, wheat, potatoes, bread, and cereals), lean meats, yogurt, fruits, and vegetables. Fatty foods and foods high in simple sugars (including juices and soft drinks) should be avoided. The American Academy of Pediatrics initially recommended gradual reintroduction of milk-based formulas or cow's milk in the management of acute diarrhea, beginning with diluted milk mixtures. Based on several studies (154), this recommendation was changed and at present the recommendation is that children with diarrhea can receive a regular age-appropriate diet, including undiluted milk but with active clinical monitoring to detect the few children who develop malabsorption and lactose intolerance (214). Lactose intolerance should be suspected if diarrheal disease severity worsens 3 to 4 days after the onset of diarrhea, and for those who are passing significant amounts of reducing sugars in their stool (154). In Thai and other Asian children with genetically determined low lactase levels, lactose-free diets seem to be better for recovery after rotavirus infection (215).

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Respiratory Syncytial Virus, Human Metapneumovirus, and Parainfluenza Viruses

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Respiratory syncytial virus (RSV), human metapneumovirus (HMPV, MPV), and the parainfluenza viruses (PIVs) are the most important causes of lower respiratory tract illnesses in infants and children. RSV was first isolated from chimpanzees with coryza in 1956 (1) but was soon shown to be the major cause of bronchiolitis and pneumonia in infants (2). RSV was named after the cell fusion that is characteristic of its growth in some continuous cultured cell lines. PIV types 1, 2, and 3 were first recovered in 1956 (3, 4) and were recognized as the major causes of croup, or laryngotracheobronchitis, in children. PIV types 4A and 4B have been recovered from adults and children with upper respiratory illnesses but historically were difficult to isolate in cell culture (5). MPV was first discovered in the Netherlands in 2001 (6) and soon thereafter was documented to be an important cause of lower respiratory tract illness in children worldwide. In older children and adults, these three viruses cause frequent reinfections that are generally mild in healthy persons, but they may cause serious disease in the very young or elderly, immunocompromised patients, and persons with underlying cardiopulmonary diseases.

VIROLOGY

Classification

RSV, PIV, and MPV belong to the Paramyxoviridae family. RSV is a member of the genus pneumovirus; MPV is a member of the genus metapneumovirus, and the PIVs are members of the genus paramyxovirus. A number of related human and animal paramyxoviruses are important respiratory pathogens, including measles (Chapter 39) and mumps (Chapter 38) viruses (Table 1; see also Chapter 40 on Zoonotic Paramyxoviruses). RSV is comprised of two subgroups that are antigenically distinct, classified as RSV/A and RSV/B (7). The subgroups are further classified into genotypes based on the variability of the distal third of the G gene; amino acid variability is up to 20% within group A and 9% in group B (8). The major difference between the subgroups is the antigenic properties of the attachment (G) surface glycoprotein protein (8), while the F or fusion surface glycoprotein remains relatively conserved (Table 2) (8). Of the three surface proteins (small hydrophobic or SH, F, and G), only the F and G surface glycoproteins evoke antibody responses that are important for conferring protection against life-threatening RSV illness.

In contrast to RSV, PIVs have no clinically significant antigenic variants within each of the recognized 5 types (1, 2, 3, 4A, and 4B) (9). Immune responses to the two PIV surface glycoproteins hemagglutinin and neuraminidase (HN) and F appear to correlate with protection against infection.

Several MPV genes including F, G, and phosphoprotein (P) have been used for subtyping, and phylogenetic analysis of these sequences have defined two major genetic subgroups of MPV, A and B, each with two minor subgroups (10, 11). The two major MPV groups (A and B) show significant genetic variability in the G gene (50%–57% nucleotide identity with 30%–37% amino acid identity between MPV/A and MPV/B subgroups) and relative conservation in the F gene (84% nucleotide identity with 94%–97% amino acid identity between subgroups) (11). F protein induces neutralizing antibodies and appears to be the major protective antigen.

Structure

The virions are pleomorphic, varying from almost spherical to filamentous in shape, and range in diameter from 150–300 nm (12). Large filamentous forms are often noninfectious because they may lack a nucleocapsid. All of these viruses have an envelope consisting of a lipid bilayer derived from the host cell with spike like membrane-anchored glycoproteins. The main elements of their structures are illustrated in Fig. 1. The lipid-containing envelope is lined by a matrix protein that surrounds the helical nucleocapsid. The diameter of the nucleocapsid of PIV is 18 nm whereas that of RSV is slightly smaller at 12 to 15 nm. The genomes are composed of single-stranded RNA with negative sense polarity but vary in size. The RSV genome consists of 15.2 x 10^3 nucleotides, the PIV genome consists of 15.5 x 10^3 nucleotides, and the MPV genome consists of 13.3 x 10^3 nucleotides.

The organization of the genome differs for the three viruses (Fig. 2). Eleven proteins are encoded by the RSV genome, two of which are nonstructural. PIVs have at least six structural proteins and one or more nonstructural proteins. MPV encodes nine proteins analogous to those of RSV but lacks NS1 and NS2. The nucleocapsid comprises the RNA bound to the nucleoprotein (N) with the P protein, the transcription processivity factor M2-1, and large L proteins that have RNA-dependent RNA polymerase activity.

The compositions and major proteins of RSV, the PIVs, and MPV are compared in Table 2. RSV, PIV, and MPV all

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TABLE 1 Animal viruses related to human respiratory paramyxoviruses

Human strain	Related animal strain[a]	Animal host(s)	Disease
PIV type 1	Sendai virus	Rodents	Pneumonia
PIV type 2	Simian virus 5	Dogs	Canine croup
PIV type 3	Bovine PIV type 3	Cattle	Shipping fever
RSV	Bovine/ovine RSV	Cattle, sheep	Shipping fever
MPV	Avian metapneumovirus (formerly turkey rhinotracheitis virus)	Turkeys, chickens	Swollen head syndrome

[a]Sendai virus of rats is closely related to PIV type 1. Simian virus 5, a cause of canine croup, is related to PIV type 2. Bovine PIV type 3 contributes to the economically important shipping fever complex of cattle and is closely related to human PIV type 3.

have an F protein in the envelope, but each has a distinct second large surface glycoprotein. PIV has an HN protein with both hemagglutinating and neuraminidase activities, while RSV and MPV have a large glycoprotein, designated the G protein, that is important for attachment but lacks these activities. RSV and MPV have a relatively conserved SH protein in the envelope that appears to form a viroporin with undefined biological function (13).

Replication

Infection is initiated by attachment of the virion to a susceptible cell by the HN, G, or F protein (14–17). The putative receptor for RSV G protein is heparin sulfate (18, 19), although recent work suggests CX3CR1 is a receptor for G on primary airway epithelial cells (20, 21), Nucleolin is a putative receptor for RSV F (22). PIV HN binds to cell surface sialic acid to initiate infection (5). MPV F binds to Arg-Gly-Asp (RGD)-binding integrins to mediate attachment and infection and is capable of entering cells by endocytosis (16, 17, 23), and viruses with mutations of the RGD motif are attenuated in rodents (24). MPV F and G proteins bind to heparan sulfate, which may serve as a receptor (15, 25). The F protein must be cleaved by cellular proteolytic enzymes to become activated and capable of fusion. The cleaved F proteins (F1 and F2) act to fuse the host cell membrane to the virus lipid envelope to allow the nucleocapsid to enter the cytoplasm. RSV, PIV, and MPV replicate solely in the cytoplasm with no requirements for nuclear processing. The virion polymerases (P+C and L) transcribe the negative-strand RNA to mRNA for production of the viral proteins (Fig. 3). The negative-strand RNA also serves as a template for synthesis of the genomic intermediate positive-strand RNA. The virion-complementary RNA is then copied into progeny negative-strand RNA. The virion is assembled with the progeny negative-strand RNA and structural virus proteins, and then released by budding from the host cell membrane.

New understanding of viral entry mechanisms of RSV and PIV is being elucidated. HPIV infects its target cells by coordinated activity of the HN receptor-binding protein and the fusion envelope glycoprotein F (26). The prefusion F undergoes a structural transformation following binding of the HN and F, inserting itself into the target cell membrane, and then changing into a postfusion structure that mediates fusion of the viral and cell membranes (26). Fusion mechanisms and machinery differ in strains grown in human airway epithelia and those adapted for *in vitro* replication. Unlike most paramyxoviruses, RSV appears to enter the cell by macropinocytosis, followed by cleavage of the second furin site of the F protein in the endocytic compartment, with subsequent fusion and release of infectious nucleocapsid (27). MPV is also capable of entering cells to initiate infection via clathrin-mediated endocytosis, as well as fusion at the cell membrane (23).

Host range

Human RSV infects nonhuman primates and, under experimental conditions, rodents, including cotton rats and mice (28–32). Disease manifestations due to RSV in primates and cotton rats have similarities to those in humans; RSV infection in mice is characterized by constitutional symptoms such as weight loss and fur ruffling with minimal pulmonary symptoms, despite histopathologic changes in the lungs. Viruses closely related to RSV cause symptomatic disease in cattle, sheep, and mice (33). Counterparts of the human PIVs are found in animals, and human PIV strains are also found in wild nonhuman primates (34) (Table 1). MPV is most closely related to avian metapneumovirus (formerly turkey rhinotracheitis virus) type C, which is an important pathogen in agricultural poultry. Although an initial report indicated MPV did not replicate in experimentally inoculated chickens and turkeys (6), another group reported that turkeys inoculated intranasally with MPV developed rhinitis associated with MPV RNA detection by RT-PCR but without confirmed productive MPV replication

TABLE 2 Proteins of RSV, MPV, and PIV

RSV		MPV		PIV	
Gene	Protein	Gene	Protein	Gene	Protein
F	Fusion	F	Fusion	F	Fusion
G	Attachment	G	Attachment	HN	Hemagglutinin-neuraminidase
M	Matrix	M	Matrix	M	Matrix
N	Nucleoprotein	N	Nucleoprotein	NP	Nucleoprotein
P	Phosphoprotein	P	Phosphoprotein	P+C	Polymerase complex
L	Large polymerase complex	L	Large (RNA polymerase)	L	Large polymerase complex
SH	Short hydrophobic	SH	Short hydrophobic		
M2-1	Nonstructural	M2-1	Nonstructural		
M2-2	Nonstructural	M2-2	Nonstructural		
NS1	Nonstructural				
NS2	Nonstructural				

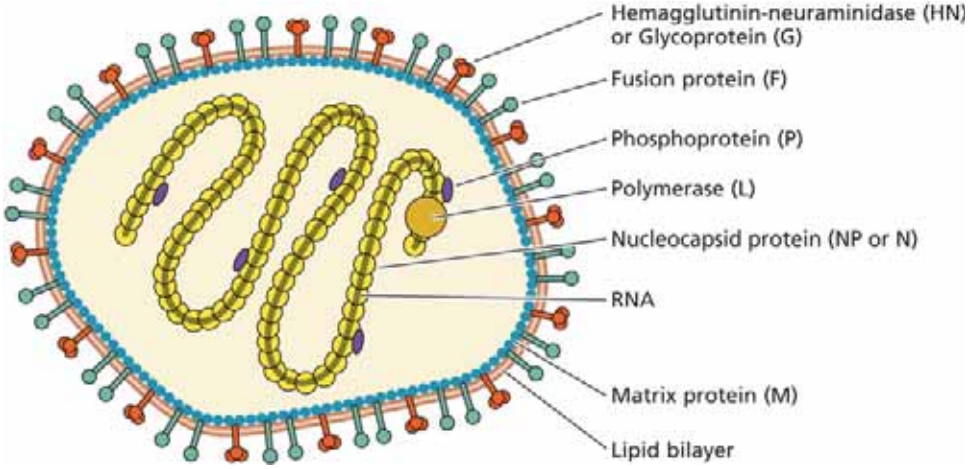

FIGURE 1 Cartoon model of the structure of viruses of the family *Paramyxoviridae* including MPV, PIVs, and RSV. Abbreviations: F, fusion protein; HN, attachment protein for PIV; G, attachment protein for RSV, MPV; M, matrix, L, large protein of the polymerase complex; NP or N, nucleoprotein; P, phosphoprotein.

(35). MPV is capable of infecting cotton rats, hamsters, ferrets, mice, and nonhuman primates under experimental conditions (36–40). MPV has also been reported to cause disease in wild chimpanzees and gorillas, likely transmitted from human tourists (41, 42, 43). Some experimental challenge studies suggest that the inoculum size is an important variable for achieving RSV and PIV infections in adults and possibly infants and children (44–46). A limitation of most animal models is that they require a large inoculum.

Stability

Because of their lipid-containing envelopes, these respiratory viruses are sensitive to ether and other lipid solvents. RSV and the PIVs differ in their lability; RSV is relatively unstable, and isolation in cell culture is enhanced by immediate inoculation into sensitive cells without freezing the specimen. By contrast, PIV are relatively stable and remain viable in a virus-stabilizing medium for up to 5 days at 4° C (15). MPV is relatively stable to repeated freeze-thaw cycles, and infectious particles can persist on nonporous surfaces for up to 6 hours (47).

EPIDEMIOLOGY

Geographic Distribution

RSV, PIV, and MPV are recognized as causes of upper and lower respiratory tract disease across all age groups worldwide. These ubiquitous pathogens are especially important causes of disease in young children. In particular, RSV infection in infants is associated with high morbidity and mortality (48, 49). Importantly, respiratory diseases remain the most important cause of mortality in young children worldwide, with an estimated 3 to 4 million deaths annually of children less than 5 years old (50).

Age-specific infection rates

Infections with all three viruses are common during early childhood (51–53). Approximately two-thirds of infants are infected with RSV and PIV type 3 during the first year of life (Table 3). Most primary infections in children occur during the first 2 years of life and are symptomatic; RSV infections are more likely to involve the lower respiratory tract, while PIV type 3 infections are more likely to produce illnesses limited to the upper respiratory tract (Table 3). Infants and young children are also commonly infected with MPV, although the mean age of hospitalization with MPV infection is approximately 6 months older (54–65). Virtually all children are infected with RSV by 2 years of age and with MPV by 5 years of age. Infections with PIV types 1 and 2 occur at a lower rate; by age 5 years 74% and 59% of children have been infected with types 1 and 2, respectively (66). Hospitalization for RSV, PIV, or MPV illness is common in U.S. children (Table 4) and elsewhere.

Severe disease due to RSV infection in children without underlying chronic conditions is most common in infants younger than 6 months of age. After primary infection, the

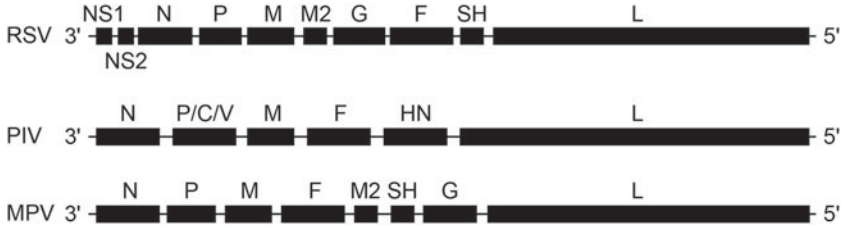

FIGURE 2 Schematic presentation of the RSV, PIV, and MPV genomes. Abbreviations: NP, nucleoprotein; P, phosphoprotein; P+C, small proteins of the polymerase complex; M, matrix; F, fusion; HN, hemagglutinin-neuraminidase; L, large protein of the polymerase complex; NS, nonstructural proteins; N, nucleoprotein; SH, strongly hydrophobic protein; G, attachment protein; M2, small envelope protein. Gene sizes are roughly to scale.

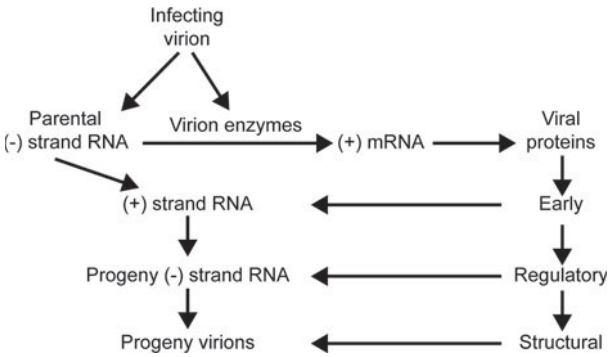

FIGURE 3 Schematic presentation of the replication strategy of viruses of the family *Paramyxoviridae*. Abbreviations: (-), negative sense; (+), positive sense.

incidence of RSV lower respiratory tract falls with increasing age (52, 67), until late adulthood, when increasing rates are again noted in the elderly (68). Immunity induced by primary infection has a limited effect on illness associated with the first reinfection, but the severity of illness is significantly reduced by the third RSV infection (52). Repeated infections in children and adults occur frequently, indicating that protection against reinfection is incomplete (52). Asymptomatic infection is more common in adults and may contribute to spread (69).

For PIVs, lower respiratory tract infection occurs most frequently in children younger than 4 years of age (51). Relatively few cases of severe disease in infants less than 4 months of age have been documented, although severe disease has been reported in premature infants or those hospitalized in neonatal intensive care units (70). Immunity elicited after infection appears to be very important in reducing the severity of disease following reinfection.

Hospitalization of children for MPV infection occurs primarily in the first year of life, although many studies report that the peak age of hospitalization for MPV is from 6 to 12 months of age and thus later than the peak age of hospitalization for RSV (54–65). MPV is rarely detected in asymptomatic children (65, 71, 72).

RSV and MPV are important causes of acute respiratory infections in adults, especially among older adults and high-risk adult populations (68, 73–80). RSV and MPV infections are more common in adults at high risk, such as those with chronic cardiopulmonary disease, immune compromise, or age greater than 65 (73, 74, 77, 78, 81–90). Medically significant PIV infections appear to be less common in adults, although there are fewer published studies (91–94). Most PIV disease in adults occurs in high-risk populations (95–100).

Reinfection

Reinfections with these viruses are common. Overall, 76% and 67% of children are reinfected with RSV and PIV type 3, respectively, during the second year of life (Table 5). Repeated infection is common but usually results in upper respiratory infection or inapparent infection, with markedly lower rates of lower respiratory tract disease (51). Only 3% of children less than 5 years old develop lower tract disease with PIV type 3 reinfection compared to 11% who have lower tract disease with RSV. Reinfection with MPV can cause upper or lower respiratory tract disease (53, 72, 101). Reinfections with these viruses also cause a sizable proportion of upper respiratory illnesses in healthy older children and adults. RSV, PIV, and MPV reinfections are common causes of hospitalization of adult patients with chronic lung conditions such as asthma and chronic obstructive pulmonary disease (86,102–104). RSV and MPV can cause serious lower respiratory tract infection among immunocompromised adults, residents of long-term care facilities, and the elderly who live in the community (73, 75, 77, 78, 94, 105–109). Of pneumonia hospitalizations and deaths in older adults, 2% to 9% are due to RSV, and 2% to 11% to MPV (76, 110).

Clinical Attack Rates

RSV, PIV, and MPV are the most important causes of acute lower respiratory tract disease in children (111–113). Almost 30% of infants have a medically attended illness due to RSV in the first year of life that is usually diagnosed as bronchiolitis or pneumonia (114). RSV-related hospitalization rates for lower respiratory tract diseases are similar in developed and developing countries (115). At least 2% of all infants are hospitalized with RSV disease, with the peak occurrence in the second month of life, though hospitalization rates are higher in high-risk groups such as premature infants (116, 117). Infants from low-income households and many Native American or aboriginal populations have a much higher risk of hospitalization than those from upper- and middle-income groups (102). The frequency of RSV lower tract disease decreases gradually during the preschool years. In a classic Houston family study, about two-thirds of children were infected during each of the first 2 years of life, with the risk of illness at least 30 per 100 children per year (51). In a Chapel Hill daycare study, the age-specific attack rate for PIV3 lower respiratory tract disease paralleled that of RSV, with annual attack rates ranging from 7 to 15 per 1000 children per year for children less than 3 years old. Boys have a greater frequency of clinical croup (118).

TABLE 3 Primary infection rate for RSV and PIV type 3 in children younger than 3 years[a]

	RSV			PIV type 3		
Age (mo)	Total no. of children	No. (%) Infected	No. (%) with LRD[b]	Total no. of children	No. (%) Infected	No. (%) with LRD[b]
0–12	125	85 (68.0)	27 (21.6)	121	75 (62.0)	13 (10.7)
13–24	34	33 (97.1)	2 (5.9)	37	30 (81.1)	8 (21.6)
25–36	1	1 (100)	0	3	2 (66.7)	0
Total	160	119 (74.4)	29 (18.1)	161	107 (66.5)	21 (13.0)

[a]Data from references 51 and 52.
[b]LRD, lower respiratory tract disease.

TABLE 4 RSV, MPV, and PIV type 3 hospitalization rates in U.S. children younger than 5 years[a]

	Incidence of hospitalization per 1000 children		
Age (mo)	RSV	MPV	PIV3
0–5	16.9	3	1.6
6–11	5.1	2	1
12–23	2.7	1	0.7
24–59	0.4	0.5	0.2
Total	3	1.2	0.5

[a]Data from references 65, 72, 126, 130, and 177.

RSV is associated with a substantial number of hospitalizations for acute respiratory infections in adults. A longitudinal cohort study over four seasons found that RSV was associated with approximately 11% of all hospitalizations for pneumonia, similar to the rate for influenza (74). The mortality of hospitalized adults with RSV (8%) was similar to the death rate for influenza (7%). Another study used a modeling approach to combine hospital discharge data with national respiratory virus surveillance data to estimate age-specific hospitalization rates for RSV and influenza (119). This study found a rate of 86/100,000 for RSV hospitalization in adults greater than age 65, less than the rate for influenza of 309/100,000. Other studies also found high rates of RSV among hospitalized older adults (79, 80, 120–124).

Rates of hospitalization, clinic visits, and emergency department visits for MPV disease are lower than that of RSV and similar to influenza (65, 79, 125). PIV type 3 infects children at an early age, but these infections are much less likely to involve the lower respiratory tract. The highest incidence of lower respiratory tract disease due to PIV types 1 and 2 occurs between 6 and 18 months of age (126). The total impact of the three PIVs combined, as reflected by admissions to the hospital, is about the same as that of RSV. Attack rates of PIV4 may be as high as those for PIV 1, but there are fewer data on PIV4 epidemiology (127). The PIVs have lower rates than does RSV in infants but higher rates than RSV in the 1-to-4-year age group (66).

The health and associated economic impacts of RSV infections are substantial and extend beyond the acute episode (128). In the Unites States approximately 2 million health care visits for RSV occur annually in children less than 5 years of age, of which 1.7 million are office visits and 580,000 emergency room visits, resulting in 60,000 to 144,000 hospitalizations with an average stay of 3.4 to 3.9 days (117). The annual direct medical cost is estimated to be $1.15 billion dollars with another $625 million due to lost income and other expenses for caregivers (129). The annual combined cost for hospitalizations due to PIV types 1, 2, and 3 in the United States is $259 million (130). Fewer data are available for MPV, but one study used the population-based burden of hospitalization for MPV (65) to estimate an annual cost for hospitalizations due to MPV at $277 million (131).

Epidemic occurrence and seasonality

Distinct seasonal patterns of infections occur for these agents in the temperate zones (51, 132). In temperate climates RSV produces annual midwinter epidemics that are characterized by bronchiolitis in infants, particularly in large urban centers, but some variations occur. For example, in the southern United States, the RSV season generally begins earlier than in other regions of the country. Epidemics that alternate between large midwinter epidemics followed by small early spring outbreaks in the following season have been reported in some countries. RSV outbreaks are less clearly delineated in tropical areas, where year-round infection is reported with or without epidemics (132). RSV/A infections usually predominate, but sometimes RSV/B infections are more prevalent, and in certain settings, RSV/B may alternate with RSV/A (7, 133). In recent years, the RSV/B/Buenos Aires genotype and the RSV/A/Ontario genotype have become the dominant RSV genotypes in circulation worldwide (134, 135). These genotypes are distinctive in that they have a 60- or 72-nucleotide insertion in the distal third of the G gene, possibly increasing viral fitness compared to other genotypes.

PIV type 1 causes croup epidemics every other year in the autumn (51). PIV type 2 infections usually follow the same pattern as PIV type 1, but the manifestations of PIV type 2 infections are milder. PIV type 3 circulation is the least predictable; infections occur in an endemic pattern most of the time but outbreaks do occur, usually in the spring, and disease is characterized by both upper and lower respiratory tract infections. PIV-3 remains the most common PIV type detected in both hospital-based and outpatient studies; either PIV-1 or PIV-4 may be the second most common PIV detected in children, depending on the year (136, 137). PIV type 4 may be detected year round (137), is associated with less severe respiratory symptoms compared with other PIVs, and is not associated with croup (127). Although PIV-4a has typically been more commonly reported, subtype 4b has been detected nearly as often in recent studies in China (138).

Different lineages of MPV frequently circulate in a community during the same winter season, although one subgroup may predominate in a given year (53, 139, 140). Viruses from each subgroup appear capable of causing severe lower respiratory tract disease; different subgroups have not been convincingly associated with varying severity of disease (141–144).

TABLE 5 Reinfection rates for RSV and PIV type 3 in children 1 to 5 years of age[a]

	RSV			PIV type 3		
Age (mo)	Total no. of children	No. (%) Infected	No. (%) with LRD[b]	Total no. of children	No. (%) Infected	No. (%) with LRD[b]
13–24	58	44 (75.9)	11 (19.0)	55	37 (67.3)	2 (4)
25–36	64	29 (45.3)	7 (10.9)	62	21 (33.9)	2 (3)
37–48	39	13 (33.3)	3 (7.7)	39	13 (33.3)	1 (3)
49–60	24	12 (50.0)	0	24	4 (16.7)	0
Total	185	98 (53.0)	21 (11.4)	180	75 (41.7)	5 (3)

[a]Data from references 51 and 52.
[b]LRD, lower respiratory tract disease.

Outbreaks of infections with these respiratory viruses are common in closed populations of young children, especially in pediatric hospital wards or large daycare centers (145). Reports of epidemic RSV disease in nursing homes (146) and homes for adolescents with developmental disabilities (147) have also been described. Similarly, numerous epidemics of PIV and MPV have been documented in pediatric wards, neonatal intensive care units, and hematology-oncology wards, as well as in long-term care facilities (70, 90, 148–153).

Epidemics of RSV or PIV can be recognized by clinical syndrome, for example, seasonal outbreaks of bronchiolitis in infants in the autumn and winter months or croup epidemics due to PIV types 1 and 2 in slightly older infants and toddlers in the autumn. PIV type 3 outbreaks tend to occur in late winter or spring. MPV generally follows the RSV season in the late winter or early spring. Many upper respiratory illnesses that occur in older children and adults during these periods are caused by reinfections with these viruses. However, in general, these viruses are not clinically distinguishable.

Transmission

Most evidence indicates that these viruses are transmitted by respiratory secretions through direct contact or via fomites (154) or by large droplet spread (155). RSV has been recovered from environmental surfaces in infected patients' rooms for up to 6 hours (156) and MPV can remain viable on nonporous surfaces for 6 hours (47). Entry likely occurs through contact with nasal mucosa or eyes, in contrast to the less permissive oral route (46). Transmission by small particle aerosols of RSV has not been proven, and if it occurs, it is an infrequent route. Clinical observations suggest PIVs and MPV are transmitted similarly to RSV (157). Although PIV types 1 and 3 have been recovered from air samples collected in the vicinity of infected patients (158), direct contact and transmission via fomites are likely to be more important. The high initial and subsequent infection rates suggest that these viruses spread readily, that reinfected persons may be infectious, and that relatively small inocula are necessary to infect.

A preschool or school-age sibling is the most likely source of RSV and PIVs in the family, with approximately half of family members becoming infected once the virus is introduced (159). On average, the time between primary and secondary infections in family members is 5 to 6 days.

Nosocomial infections

RSV, PIVs, and MPV are common causes of nosocomial infections, contributing to significant morbidity and mortality in pediatric wards, and can involve health care workers and patients both as sources and transmitters of disease (148, 160–164). PIV outbreaks may be more difficult to bring under control due to prolonged asymptomatic shedding, particularly in young children and immunocompromised hosts (165). Nosocomial RSV and PIV infections in adults with leukemia and bone marrow transplants are associated with a high mortality (83, 164, 166). The transmission of identical strains of RSV from the outpatient setting into the hospital has been documented, demonstrating the importance of infection control measures (162). Nosocomial transmission of MPV also occurs, with outbreaks in both inpatient and outpatient units. In one study, 15 patients were diagnosed with MPV within 7 weeks in a tertiary care cancer unit (163). Molecular subtyping revealed infection with genotype A2a virus, implicating nosocomial transmission. Four patients (26.6%) died from MPV-associated pneumonia and consequent multiorgan failure.

PATHOGENESIS

Virus replication patterns

Infants and young children shed infectious RSV in nasal secretions for a mean of 7 days, with a maximum of 3 weeks (167). Nearly all hospitalized infants shed RSV during the first 7 days of hospitalization, and the majority still shed virus at the time of discharge (168). High viral titers, 10^5 to 10^7 plaque-forming unit (PFU) per ml, can often be found in secretions from the upper and lower respiratory tract in young children (168, 169). In immunocompromised adults with uncomplicated infection, very low titers of RSV ($\sim$35 PFU/ml) are detected in the upper respiratory tract, making rapid antigen tests less sensitive in adults than in children (169). Another study using RT-PCR detection of RSV in nasal swab and sputum specimens found that outpatient and hospitalized adults with RSV illness had modest amounts of detectable virus ($\sim$2–3 $\log_{10}$ PFU equivalents/ml) for a mean of 10 days and a maximum of 13 days (170). Viral detection was more prolonged in adults age 65 or older, and hospitalized adults had higher viral loads than outpatients. Another study of hospitalized adults also found that viral load correlated with disease severity (171). Prolonged replication for weeks to months may occur in immunocompromised hosts (81, 169).

The duration of viral replication for PIVs is approximately a week, although this is variable and dependent on age, number of prior infections, and severity of infection (157). PIV type 3 may be shed for up to 4 weeks, and 17% of specimens from infected children are positive during the third week after illness onset (172). Some children may excrete PIV 6 days before the onset of illness, and prolonged PIV detection may occur in children with primary infection and adults with underlying chronic lung disease (172, 173). Prolonged viral shedding (mean=4 weeks) occurs in immunocompromised children and adults and is associated with malignancy, organ transplantation, congenital or acquired immunodeficiency, or prolonged steroid treatment (164). Persistent detection of PIV RNA in asymptomatic immunocompromised patients for several months has been noted using more sensitive molecular methods (165).

MPV replication in immunocompetent children and adults is limited to 1 to 2 weeks (174, 175), although it can be prolonged in immunocompromised persons (163, 174, 175).

Organ specificity

In the immunocompetent host, RSV, MPV, and PIV replication is restricted to the respiratory epithelium. Viremia or isolation of RSV, PIV type 3, or MPV from cerebrospinal fluid has been reported rarely. All these viruses spread primarily from cell to cell in the upper respiratory tract and at times progress to the lower respiratory tract.

Histopathology

The pulmonary histopathology of bronchiolitis has been described primarily from infants and young children who have succumbed acutely from RSV infection (176, 177). Limited data are available for PIVs and MPV, although the histopathology appears similar to that of RSV (177–180). The earliest lesion to develop within 24 hours of onset is necrosis of the bronchiolar epithelium with denudation of the ciliated epithelial cells. This is followed by migration of lymphocytes into affected tissues, resulting in peribronchiolar infiltration (Fig. 4). The submucosal and adventitial tissues become edematous and accompanied by increased secretions from mucus-producing cells. Plugs consisting of mucus, cellular debris, fibrin strands, and DNA-like

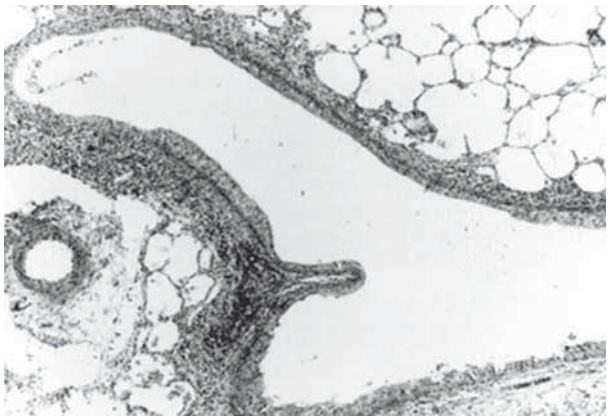

FIGURE 4 Characteristic histopathologic changes of the airways of an infant with bronchiolitis due to RSV infection. Note the peribronchial mononuclear cell inflammation, relatively normal alveoli, and focal ulceration on top with regenerative epithelial changes. Hematoxylin and eosin stain. Magnification, 45x.

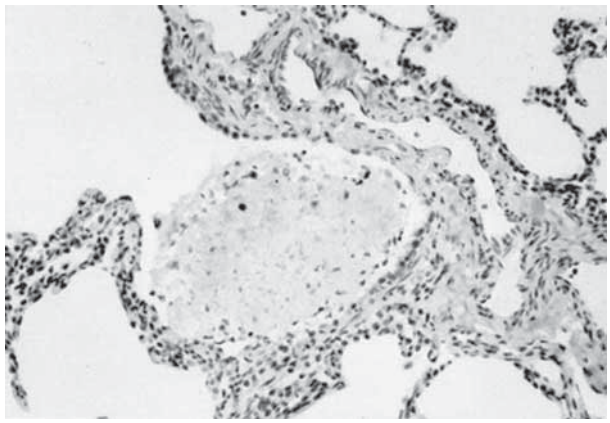

FIGURE 5 Histopathologic features of resolving RSV bronchiolitis in an infant. Note the plug of mucus and cellular debris in a terminal respiratory bronchus. Hematoxylin and eosin stain. Magnification, 144x.

materials occlude the smaller bronchioles. Activated neutrophils, a major cellular component in the airway lumen in bronchiolitis, release neutrophil extracellular traps, a network of DNA-containing antimicrobial proteins, that probably contribute to airway occlusion (181). However, polymorphonuclear cells are not common in the bronchial epithelium and parenchyma of the lung. Fatal RSV infection has been characterized by viral antigen detected primarily in cellular debris obstructing the lumen of the small airways and in multinucleated cells lining the bronchiolar lumen, near absence of CD-8 positive lymphocytes and natural killer cells, and enhanced expression of apoptosis markers (182, 183). The major pathologic features are air trapping with distension of some segments or obstruction with alveolar collapse in bronchiolitis and extension of necrosis into the parenchyma with interstitial pneumonia. Histologic recovery is slow, beginning with regeneration of the basal epithelial layer within 3 to 4 days and exuberant regeneration of the ciliated epithelial cells starting at the end of the second week (176). The plugs of cellular debris are eventually resorbed (Fig. 5).

Interstitial inflammation with mononuclear cell infiltration is the rule in RSV and PIV pneumonia (Fig. 6) (177, 184). The epithelial cells are flattened, with loss of cilia. The subepithelial tissues of bronchiolar and interalveolar walls are thickened due to mononuclear cell infiltration. Subsequently, significant epithelial necrosis, intense mononuclear inflammatory response extending from the smaller bronchioles into alveoli, hyaline membrane formation in alveolar spaces, and edema of interalveolar walls may occur. Histologic differences observed between bronchiolitis and pneumonia may represent a continuum of disease possibly related to the size of the viral inoculum, with higher concentrations of virus resulting in pneumonia (184).

MPV has been detected almost exclusively in respiratory tract specimens, suggesting that its replication is limited to respiratory epithelia. In lung transplant recipients infected with MPV, both acute and organizing lung injury occur, with diffuse alveolar damage and cytoplasmic inclusion bodies (185). Bronchoalveolar lavage fluid from immunocompromised patients contains sloughed, degenerated epithelial cells with eosinophilic cytoplasmic inclusions, multinucleated giant cells, and histiocytes (186). Because available histopathologic evidence comes from lung biopsies and bronchoalveolar lavage specimens from patients with underlying immunodeficiency or malignancy, these findings may not reflect what occurs in otherwise healthy humans. The pathologic features of MPV infection in mice, cotton rats, and macaques include disruption of respiratory epithelial architecture, sloughing of epithelial cells, loss of ciliation, and inflammatory infiltrates (36, 37, 187). Histopathologic changes or evidence of infection was not detected in any other tissues. In macaques and cotton rats, viral antigen is localized almost exclusively at the apical surface of ciliated respiratory epithelial cells. The limited histologic data from human autopsies suggests that syncytia can be found *in vivo*, especially among immunocompromised persons, but are less common than in measles pneumonia (161, 178, 183, 188–190).

IMMUNE RESPONSES

Innate response: cytokines and inflammatory mediators

Upon virus infection of the respiratory epithelium, the innate immune response is the first line of host defense.

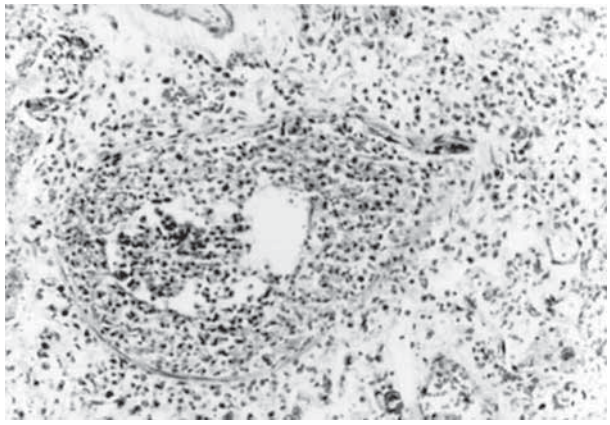

FIGURE 6 Histopathology in an infant with congenital immune deficiency and RSV pneumonia. Note the plugging of a terminal bronchus by cellular debris and parenchymal infiltration with necrosis. Hematoxylin and eosin stain. Magnification, 72x.

Mannose-binding lectin, surfactant proteins, and other collectins act as opsonins with activity against RSV and likely MPV and PIVs. Signaling receptors such as Toll-like receptor 4, which recognizes the fusion protein of RSV, can activate the NF-κB signaling pathway, inducing the expression of cytokines and costimulatory molecules. Gene polymorphisms of pattern-recognition receptors (Toll-like receptor 4 and surfactant protein D) and of downstream-induced antiviral molecules (interleukin 4 [IL-4], IL-8, IL-10, and tumor necrosis factor alpha [TNF-α]) and other innate immune genes have been associated with severe RSV infection (191–193). Cytokine production is depressed in infants and children compared with adults and may account for their less-than-optimal immune response (194).

Unlike several other respiratory viruses, RSV infection is notable for the induction of little or no local interferon (195, 196). The host interferon response to RSV is suppressed by RSV nonstructural proteins NS1 and NS2. *In vitro* studies of PIV3 have demonstrated the induction of interferon-gamma (IFN-γ) and RANTES (regulated on activation, normal T-cell expressed and secreted) protein in human nasal epithelial cells (197), correlating with human studies demonstrating detectable levels of interferon in 30% of children with PIV infection (196). Interferon induction following PIV infection has been hypothesized to be associated with diminished shedding of the virus (196). The P/C/V gene of PIV type 1 inhibits interferon response by inhibiting interferon regulatory factor-3 activation and subsequent interferon production (198, 199).

The clinical significance of the cytokine profiles associated with severity of RSV and other respiratory viruses is emerging. Although it was thought that the inflammatory response generated against RSV contributed substantially to the development of respiratory disease, recent data suggest that a robust early inflammatory response is critical in the control of viral replication and mitigation of respiratory disease (182, 198, 199). Each respiratory virus has a distinct cytokine profile, even though the clinical presentation of disease is similar (182, 198, 199).

IL-2 and IFN-γ are the predominant RSV-stimulated cytokine responses of memory T cells from young children exposed to one RSV season, older children with one or more prior RSV infections, and adults (200, 201). Some children also develop an increase in IL-5 mRNA. RSV-specific-memory T cells appear to have a dominant TH1 response and are similar for different age groups. TNF-α and IL-8 are produced by RSV-infected alveolar macrophages. These cytokines attract neutrophils and macrophages to the site of infection (202). IL-6, a promoter of mucosal B lymphocytes and immunoglobulin A (IgA) production, and TNF-α are detected in the nasal secretions and tracheal aspirates of most infants and young children with acute primary RSV infection, although peak concentrations are not correlated with the peak of mucosal RSV-specific IgA responses (203). Elevated nasal levels of IL-6 and macrophage inflammatory protein 1α (MIP-1α) are more common in hospitalized adults (170).

Various mediators of inflammation, in particular degranulation products of mucosal mast cells and eosinophils, have been associated with severity of involvement in bronchiolitis. Histamine and leukotrienes C4, D4, and E4 are the major mast cell mediators responsible for constriction of smooth muscle, increased mucus production, and increased vascular permeability. In infants with RSV and PIV type 3 bronchiolitis, elevated levels of histamine, leukotriene C4, and eosinophilic cationic protein have been detected during the acute phase of the disease and have been associated with wheezing and hypoxemia in RSV-infected infants (204–207).

Increased levels of IL-8 and decreased levels of RANTES have been reported in nasal secretions of MPV-infected children compared with those of RSV-infected children (208). Children infected with MPV appear to have significantly lower levels of IL-1β, IL-6, IL-8, IL-12, and TNF-α as compared with infants infected with RSV or influenza virus (199). MPV-infected children have lower levels of nasal IFN-γ and a reduced Th2 bias compared to children with influenza RSV (209). MPV infection of human dendritic cells (DCs) *ex vivo* (210) induces production of TNF-α and IL-6 in myeloid DCs and IFN-α in both myeloid and plasmacytoid dendritic cells.

Humoral, cell-mediated, mucosal immune responses

Humoral immune responses of infants hospitalized following primary RSV infection consist of virus-specific IgM antibodies that may persist up to 10 weeks after illness and virus-specific IgG and IgA antibodies that are produced within the second week, peak by 3 to 4 weeks, and decline to low or nondetectable levels by the following RSV season. Reinfection with RSV results in a rapid increase in all three classes of antibody. Age-specific and preexisting virus-specific maternal antibodies appear to influence the development of serum antibodies to the F and G surface glycoproteins and serum neutralizing antibodies in infants undergoing primary RSV infection (211). Older infants develop stronger protein-specific and functionally active neutralizing antibody responses after primary RSV infection. However, the antibody response to primary infection appears to be low avidity regardless of age (212). Primary infection with RSV/A virus elicits antibodies that cross-react with RSV/B virus (213). In contrast, primary RSV/B infection induces cross-reactive antibodies to RSV/A virus less efficiently. IgG responses to F and G proteins after primary RSV infection are predominantly IgG1 and, less frequently, the IgG3 subclass, a finding consistent with the protein domain bearing the dominant antigenic sites (214). In contrast, adults respond to the heavily glycosylated G protein with IgG1 and IgG2 subclass antibodies, with a dominant IgG1 subclass response to the less glycosylated F protein (215). The dominant IgG antibody response induced by natural infection to the F protein appears to be directed to the pre-fusion conformational form rather than the post-fusion form (216). The prefusion and post-fusion F conformations have both unique and shared antigenic sites, including a shared antigenic site that is the target for palivizumab and motavizumab (217). The structure of MPV F protein demonstrates a novel antigenic site that has a likely analogue on RSV F (218).

Antibodies directed to the G protein most likely neutralize the virus by preventing attachment of virus to cells, while antibodies directed to the F surface glycoprotein neutralize the virus by preventing virus-cell fusion and inhibiting cell-to-cell spread (219, 220). Other effector mechanisms mediated by IgG1 and IgG3, such as complement-enhanced neutralization and antibody-dependent cell cytotoxicity, help clear virus and virus-infected cells; this function has been suggested for non-neutralizing antibodies directed to the SH surface protein (221). Since RSV is restricted to the mucosa, the antibodies present in the lower and upper respiratory tract are important for disease prevention. In adults, concentrations of both IgG1 and IgG2 in serum and the terminal airways are similar (48).

Infection with PIVs induces both humoral and cellular immune responses in infected humans, including local and systemic IgG and IgA responses, and specific T-cell responses. PIV primary infection does not confer durable immunity, although immunity is usually sufficient to restrict virus replication from the lower respiratory tract and prevent severe disease following reinfection. Mucosal IgA levels correlate with protection from replication of PIVs in adults (222). Neutralizing antibodies against the HN and F protein are critical for long-term protection (223). Cellular immune responses are thought to play an important role in clearing RSV and PIV infections and preventing lethal disease. As noted above, persons with deficient cellular immunity may shed virus for months (51, 83) and infection may progress to fatal lower-tract disease (89, 161, 224).

The role of the cytotoxic T lymphocytes (CTL) in RSV, PIV, and MPV lung disease has not been fully elucidated in humans. RSV-specific CTL responses occur in a minority of infants with RSV bronchiolitis, and the highest responses occur in children with mild lung disease (225). Infants who develop RSV-specific CTL responses after RSV lower respiratory tract illness in the first year of life appear less likely to develop a lower respiratory tract illness in the second year of life (201). RSV-specific $CD8^+$ cytotoxic activity in vitro correlates with RSV-induced INF-γ and inversely with IL-4 production by CTL (201). In adults, RSV-specific CTL recognize the N (nucleoprotein) protein, the SH protein, the F protein, the M protein and, poorly, the M2 protein but not the G protein (226, 227). In elderly adults, development of severe RSV infection may be due to low numbers of RSV-specific $CD8^+$ memory T cells that maintain effector function (228). RSV-specific CTL appear to be important for recovery from infection, and their maintenance is critical for the control of subsequent RSV infections. Humans develop a $CD8^+$ T cell response to MPV, but the contribution of these cells to immunity and disease in humans is unknown (229). Mouse studies show that T cells are required to clear MPV infection and memory T cells contribute to protection against reinfection (230–232). The finding of lymphopenia as an additional risk factor for severe disease among immunocompromised hosts supports the importance of T cells in viral clearance (88, 90, 233, 234).

Impairment of T-cell function appears to be a common denominator in children with prolonged shedding due to RSV, MPV, and PIVs (161, 235). MPV and other respiratory viruses induce pulmonary $CD8^+$ T-cell functional impairment, mediated by the inhibitory receptor programmed cell death-1 (PD-1); this functional impairment contributes to delayed viral clearance and reinfection in animals and may facilitate reinfection in humans (40, 231).

Secretory antibodies found in the nasal secretions consist of IgA, IgM, IgG, and IgE classes and are directed to at least the major surface glycoproteins of these viruses (236, 237). The rate and magnitude of IgG and IgA secretory antibody responses to RSV are lower in younger than older infants. With primary infection, secretory IgG and IgM antibodies peak between 8 to 13 days, while secretory IgA peaks between 14 to 28 days after onset of illness. After 2 months, infants have low or nondetectable levels of virus-specific secretory antibodies of all three immunoglobulin classes. Primary infections with PIVs result in low, transient virus-specific IgA responses in secretions (238).

The production of virus-specific IgE antibody has been associated with RSV disease severity (205, 239). During primary RSV and PIV infections, secretory virus-specific IgE antibody has been detected primarily in infants with bronchiolitis. Virus-IgE complexes may induce mast cells to release mediators that are responsible for smooth muscle constriction, increased mucus production, and vascular permeability.

Correlates of immune protection

Infants with high levels of maternally acquired RSV- and PIV type 3–specific antibodies are better protected against lower respiratory tract illness than those with lower levels (51, 52). The level of neutralizing antibody in cord blood directly correlates with age at which primary RSV infection occurs and is inversely related to the severity of illness. The risk of reinfection in children and adults is also inversely related to the level of serum neutralizing antibodies for RSV and MPV (51, 52, 240, 241).

Human challenge models have increased understanding of the kinetics of illness following virus exposure and of the immunologic factors relevant for protection (43, 46, 242). Experimental challenge studies in adult volunteers show that serum virus-neutralizing antibody and nasal IgA titers correlate with protection against infection, although in one study serum and nasal antibody were not correlated and thus may be independent correlates of protection (242). However, symptoms, peak virus titer, and duration of viral shedding do not correlate with preexisting antibody titers among adults who have been successfully infected (43, 46, 242).

Other humoral correlates of immunity against RSV and PIV infections are IgG antibodies directed to the F surface glycoprotein and to the homologous G (HN for PIV) surface glycoprotein of the infecting virus strain (240, 243–245). However, even in the presence of high levels of virus-specific antibodies, primary infection and reinfection can occur (51, 52). For PIVs, serum antibodies to both HN and F proteins confer protection (246, 247).

CLINICAL MANIFESTATIONS

Respiratory Syncytial Virus

Any respiratory virus may be associated with a wide spectrum of illness severity ranging from inapparent infection or mild afebrile upper respiratory illness to severe and fulminating pneumonia. However, RSV and PIVs produce distinct clinical syndromes that are hallmarks of infection with these viruses, especially during epidemics. These viruses may also be associated with severe illness in a variety of immunocompromised hosts (see below).

Children

The characteristic illness caused by RSV in infants is bronchiolitis manifested by expiratory wheezing, air trapping, nasal flaring, subcostal retractions and, sometimes, cyanosis (Table 6). RSV is the most common virus associated with bronchiolitis, detected in approximately 70% of children with bronchiolitis during disease epidemics (49). Fever is not a prominent finding; approximately 50% of infants with RSV disease seen at the hospital will have moderate or greater elevations in temperature. Coryza and cough are common presenting symptoms. Radiographic pneumonia is common with primary RSV infection, and infants may have signs and symptoms of both clinical conditions simultaneously. The clinical presentation may depend on the proportion of the small airways partially occluded by the inflammatory process. This results in expiratory wheezing and air trapping, whereas subsegmental atelectasis results

TABLE 6 Clinical manifestations of RSV, PIV, and MPV infection at various ages

Infants
Bronchiolitis
Pneumonia
Croup
Asthma exacerbation
Upper respiratory tract infection
Otitis media
Older children and adults
Upper respiratory tract infection
Croup
Laryngitis
Bronchitis
Asthma exacerbation
Pneumonia (elderly)
Chronic obstructive pulmonary disease exacerbation

from complete occlusion of the small airways. Radiographic findings depend upon the same factors. Partial occlusion of the bronchioles results in hyperaeration and flattening of the diaphragms (Fig. 7), while complete occlusion results in atelectasis that may be lobar, usually involving the right middle or right upper lobe (Fig. 8).

Hypoxemia is a common finding in infants with lower respiratory tract disease (125). Apnea occurs in about 20% of hospitalized young infants with RSV illness (248) and may be the presenting sign of RSV in premature babies or infants less than 3 months old. Apnea occurs in approximately 5% of children hospitalized with bronchiolitis, generally within the first 2 days of hospitalization (249). Oxygen saturation is lower on average in infants with apnea, even if other signs of severe disease such as subcostal retractions are absent. Infants with apnea tend to be younger, to be born before 37 weeks' gestation, and to have a history of apnea of prematurity. Some deaths in infants who die unexpectedly at home may be a result of RSV infection accompanied by apnea. Interstitial pneumonitis is not commonly recognized with RSV infection, but cases are overrepresented in autopsy series (177). Therefore, the recognition of interstitial involvement should alert the caregiver to the need for repeat clinical assessment.

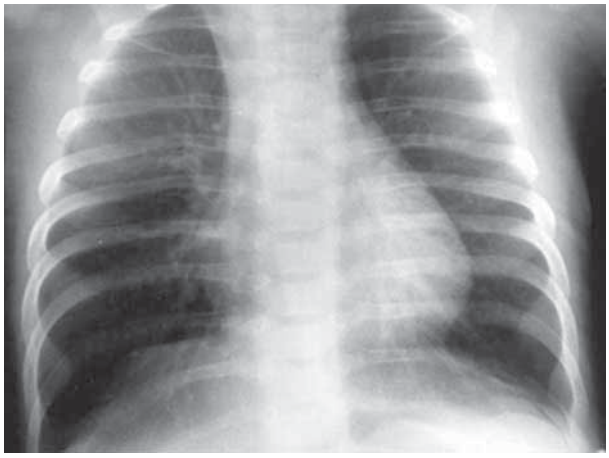

FIGURE 7 Chest radiograph of an infant with bronchiolitis due to RSV. Peribronchial thickening with hyperaeration and flattened diaphragms bilaterally are evident. Reprinted from Reference 240 with permission.

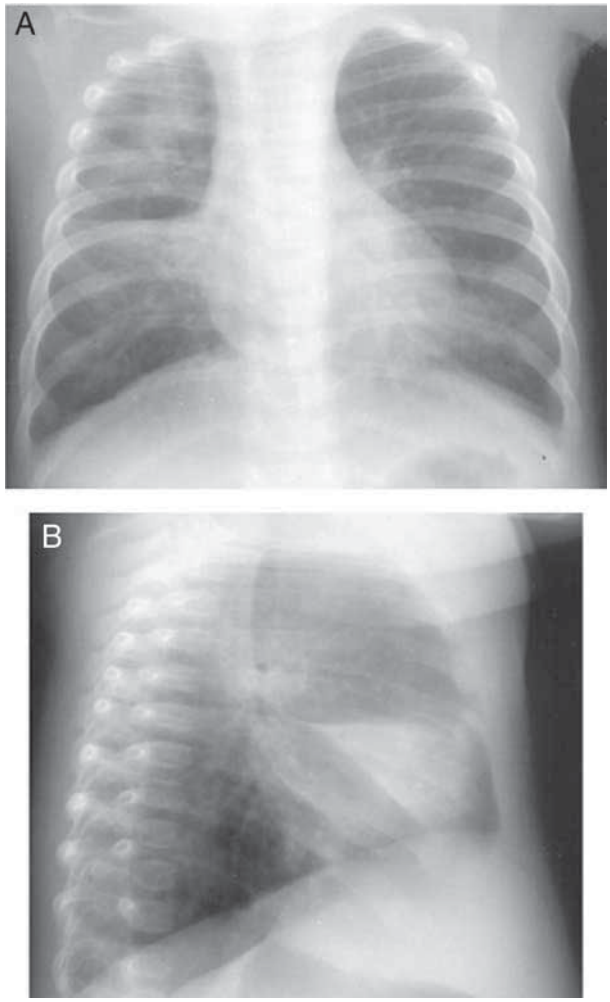

FIGURE 8 Posterior-anterior (A) and lateral (B) chest radiographs of an infant with bronchiolitis and pneumonia due to RSV. Bilateral perihilar and peribronchial infiltrations and partial or segmental atelectasis of the right middle and right upper lobes are evident. Reprinted from (240) with permission.

Acute otitis media (AOM) is a frequent complication of RSV infection in children, with rates up to 60% in some studies. RSV is commonly detected with bacteria in middle ear fluid samples (250). Severity decreases with age and following repeated infections, such that mild upper respiratory tract disease is typical of RSV infection in school-age children (115).

Adults

The common clinical presentation of RSV infection in otherwise healthy adults is similar to that reported for older children. Initial symptoms commonly include upper respiratory tract complaints like nasal congestion, cough, sore throat or hoarseness, earache, and low-grade fever (Table 6). Adults may have minimal or no symptoms and still have RSV isolated from respiratory secretions, or they may be symptomatic with sore throat, bronchitis, or wheezing. In

adults with underlying pulmonary disease, RSV infection may manifest as increased dyspnea with or without hypoxia and may be accompanied by cough, fever, nasal congestion, and wheezing (68). The clinical presentation of disease and morbidity and mortality caused by RSV and MPV in the noninstitutionalized elderly population is similar to that observed with influenza A infection (73, 78, 80). In elderly persons, clinical features with RSV infection are often indistinguishable from those of influenza, with the possible exception of a lower frequency of high-grade fever and higher frequency of wheeze in RSV (75).

Parainfluenza Virus

Prospective studies of children indicate that primary infection with PIV is usually symptomatic but often mild (51, 127). In children followed during the first two years of life, nearly one-third of primary infections result in lower respiratory tract disease, but only 5% of primary infections resulted in illnesses for which medical care was sought (127, 251). The classic syndrome for PIV in children is croup, or laryngotracheobronchitis. Croup is manifested by fever, hoarseness, and a barking cough in a child usually between 6 and 18 months of age. Severe narrowing of the subglottic area of the trachea may progress to cause inspiratory stridor (Fig. 9). Observation in the hospital may be required for children with severe stridor, so that the airway can be secured. PIV type 1 is the major cause of croup, though PIV type 2 also is associated (252). PIV type 3 causes sporadic croup but also causes bronchiolitis and pneumonia in young infants, at a lower frequency than RSV (Fig. 10). PIV type 4 disease is not associated with croup and typically presents with nonspecific respiratory symptoms that may progress to bronchiolitis, paroxysmal cough, or lower respiratory tract disease with hypoxia (253).

PIV disease in adults commonly presents as a relatively nonspecific upper respiratory tract illness, with rhinorrhea, nasal congestion, and hoarseness commonly noted. Reinfection with PIV is a common cause of serious morbidity in adults with chronic lung disease (102). In immunocompromised pediatric and adult patients, PIV infection typically presents with low-grade fever and upper respiratory tract symptoms that may persist or rapidly progress to lower respiratory tract disease with cough, wheezing, and hypoxia.

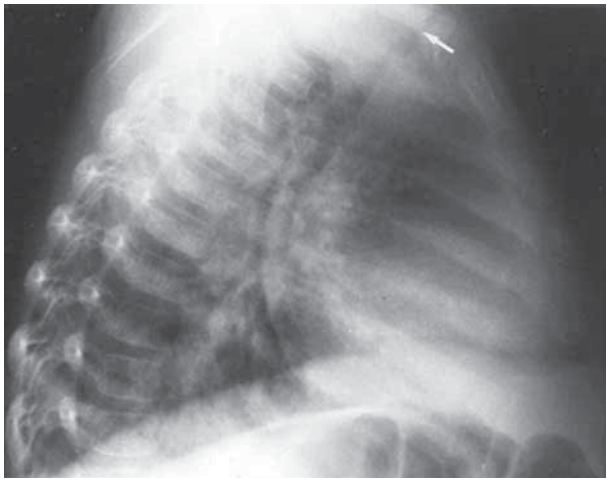

FIGURE 9 Chest radiograph of a child with croup due to PIV type 1. Peribronchial thickening, scattered infiltrates, and marked narrowing of the superior portion of the trachea (arrow) are evident.

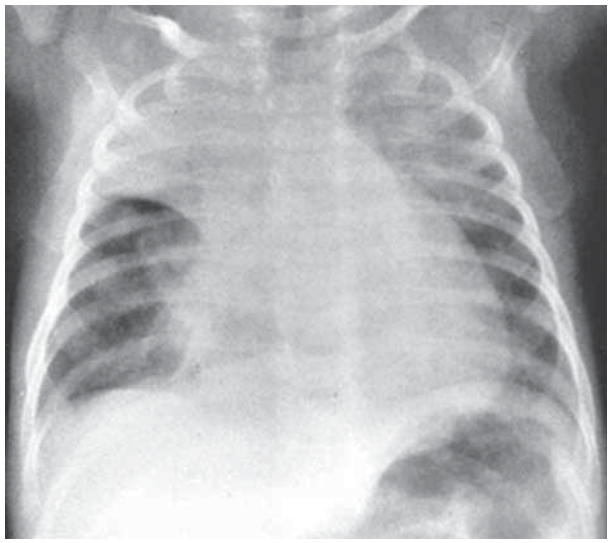

FIGURE 10 Chest radiograph of an infant with pneumonia due to PIV type 3. Right upper lobe atelectasis and multilobar infiltrates are evident.

Human Metapneumovirus

Children with MPV infection typically present with upper respiratory symptoms such as rhinorrhea, cough, and fever, similar to those with RSV and PIV (Table 6). Conjunctivitis, vomiting, diarrhea, or rash are occasionally reported but are not prominent in most studies. The lower respiratory tract syndromes most frequently associated with MPV are bronchiolitis, croup, pneumonia, and asthma exacerbation. These illnesses are neither clinically nor radiographically distinct from the same clinical syndromes caused by other common respiratory viruses (Fig. 11). Reinfections with MPV are more likely to be limited to the upper respiratory tract in otherwise healthy children (53, 72). MPV is associated with a substantial proportion of AOM in children, and viral RNA has been detected in middle ear fluid from patients with AOM (254, 255).

FIGURE 11 Chest radiograph of a six-month-old infant with bronchiolitis due to MPV. Peribronchial thickening and hyperinflation are evident. From *Human Metapneumovirus and Lower Respiratory Tract Disease in Otherwise Healthy Infants and Children*, Williams JV, Harris PA, Tollefson SJ, Halburnt-Rush LL, Pingsterhaus JM, Edwards KM, Wright PF, Crowe JE, Jr., *N Engl J Med* 350:443–50. Copyright © 2004 Massachusetts Medical Society. Reprinted with permission.

Infections in Immunocompromised Patients

Immunocompromised children and adults are vulnerable to severe RSV, PIV, and MPV infections (81, 87–89 105, 161, 165, 256, 257). RSV and PIV type 3 infections are especially devastating for infants with severe combined immunodeficiency syndrome (SCID)(208, 258) in whom persistent viral shedding and the development of progressive pneumonia occur. Children or adults who acquire RSV or MPV during or shortly after chemotherapy for malignancy also may have severe, life-threatening disease (81, 89). Children with human immunodeficiency virus (HIV) infection have a higher rate of pneumonia, decreased likelihood of wheezing, prolonged viral carriage with intermittent disease, and increased morbidity following infection by one of these viruses. Although the clinical course is generally not fulminant in HIV-positive patients when good supportive care and antiretroviral therapy are available (235, 259), the overall mortality of these patients in developing countries is substantial, with significantly higher rates of lower respiratory tract disease and mortality (7.5% vs. 0%) in HIV+ versus HIV- children (257, 260).

Hematopoietic stem cell transplant (HCT) recipients of all ages may have a more fulminant course following RSV or PIV infection, particularly if infection occurs around the time of transplantation (82, 224, 261). Adults with leukemia (262), profound chemotherapy-induced myelosuppression (262, 263), and solid-organ transplant recipients, particularly lung and pediatric heart recipients, are also at risk of fatal outcome. Initial clinical symptoms related to RSV infection in these patients are similar to those in immunocompetent persons, but upper respiratory tract infection progresses more often to lower respiratory tract disease, with likelihood of progression related to immune status (83, 88, 100, 166, 264). Risk factors for disease progression include lack of engraftment, decreased lymphocyte count, and older age (265). Evidence of pulmonary infiltrates on chest radiograph may be delayed or absent in patients with severe neutropenia but may become apparent following immune reconstitution or on chest computed tomography or magnetic resonance imaging (266). Over 1 to 2 weeks, lower respiratory tract involvement may become evident by increasing respiratory distress, worsening hypoxia, and, frequently, the need for assisted ventilation. Recovery following assisted ventilation for RSV and MPV pneumonia occurs but remains uncommon despite advances in supportive care. Quantitative bronchoalveolar lavage (BAL) viral load has not been associated with mechanical ventilation or death for RSV, PIV, or MPV in adult patients following HCT; however, the detection of respiratory virus RNA in serum has been associated with fatal outcomes (267).

Many immunocompromised adults with PIV infection first present with symptoms of mild upper respiratory tract disease, but in contrast to RSV, influenza, and MPV infections, detection of PIV-1 and -3 in asymptomatic HCT recipients is relatively common, reported in 35% of 17 infected patients in one prospective study (165). Fewer than half of PIV-infected immunocompromised patients have a fever. In severely immunocompromised patients, such as allogeneic HCT recipients less than 100 days post-transplant, PIV type 3 is the most common PIV subtype detected, reported in 80% of 544 HCT recipients with PIV (100). In all PIV-infected transplant recipients, infection may progress to lower respiratory tract disease, with more serious disease linked to supplemental oxygen requirement, low monocyte counts, and high-dose (>2 mg/kg/day) steroid use. The detection of PIV in BAL or other lower respiratory tract specimens is associated with decreased survival overall in HCT recipients (100). Higher pretransplant PIV-3 antibody levels are not protective against severe sequelae (268). Concomitant infections with other viruses or fungi or severe graft-versus-host disease are relatively common in adult patients with PIV pneumonitis (269, 270).

MPV also causes severe infections in immunocompromised hosts. Fatal infection attributed to MPV has been reported in cancer patients, and MPV is a relatively common cause of acute respiratory infection in children and adults with malignancy and HCTs or organ transplants (87, 88, 256). Risk factors for severe MPV infection include lymphopenia (88, 89, 163).

Complications

AOM is the most common complication of RSV, PIV, and MPV infection (271). The virus infection causes dysfunction of the Eustachian tube resulting in negative pressure in the middle ear. Normal clearance mechanisms for bacteria that reside in the nasopharynx are disrupted, and purulent middle ear infection may result. Acute sinusitis may develop by the same pathogenetic process.

Bacterial pneumonia may complicate lower tract infections due to these viruses, although the risk following RSV infections is low, as determined by studies of hospitalized children in the United States and Europe (272, 273). Recent studies support an association of acute RSV infection enhancing the incidence of pneumococcal pneumonia and or invasive pneumococcal disease within the 30 days after RSV infection (274–276). Superinfection with pneumococci and staphylococci are more common after PIV infections (277). Higher rates of serious bacterial pneumonia have been seen in HIV+ children in prospective studies carried out in HIV-endemic areas in Africa (260). Outbreaks of bacterial tracheitis have been reported in the past with PIV type 1 epidemics (278).

PIV2 and PIV3 have been associated with parotitis by isolation of the virus from oral swabs, although in some of these cases convalescent sera demonstrated seroconversion to mumps virus (279–281).

PIV2, PIV3, PIV4, and MPV have been associated with aseptic meningitis and encephalitis, with detection of the virus in cerebrospinal fluid (280, 282–287). However, it appears that this is a rare manifestation of infection with these viruses.

Asthma

The relationship between the development of reactive airway disease and RSV infections is intriguing (288, 289). Children with RSV bronchiolitis in infancy have a high risk of recurrent wheezing illness during the first decade of life. Longitudinal studies of pulmonary mechanics after RSV bronchiolitis show persistent abnormalities. Prophylactic use of palivizumab, a monoclonal antibody to prevent RSV hospitalization, in infants has been associated with a lower incidence of physician-diagnosed asthma and recurrent wheezing in the subsequent 2 years of life (290). Additional intervention trials have strengthened the potential causality of early virus infection and recurrent wheezing (291, 292), but establishing a causal relationship to childhood asthma will require longer studies. RSV, PIV, and MPV infections are frequently associated with asthma exacerbations in older children and adults, and prevention of these infections in

this high-risk group could significantly reduce morbidity (102, 104, 293).

A causal relationship between MPV infection and development or exacerbations of asthma is currently unproven. One study of outpatient children did not find an association between MPV and asthma exacerbations (294), while another found a highly significant association between MPV and the diagnosis of acute exacerbation of asthma (72). MPV infection has been detected in 8.9% of children hospitalized for wheezing and in 6.9% of adults hospitalized for asthma exacerbations (293, 295). One study reporting a strong association between infantile MPV bronchiolitis and asthma at age 5 years (296) requires confirmation, in part because of the difficulty of making the diagnosis of asthma during infancy, when acute wheezing frequently is associated with viral infections.

Clinical diagnosis

Epidemiological and clinical features including age, season, and clinical findings are helpful in determining the etiology of disease, particularly during relatively discrete winter outbreaks. However, because the viral seasons and symptoms can overlap, laboratory testing is required to confirm the diagnosis. The clinical findings of RSV in immunocompetent adults are nonspecific, and definitive diagnosis requires laboratory testing. Moreover, infection with more than one virus may be present. Because clinical care in young children is generally supportive, routine viral testing for all children with bronchiolitis is not currently recommended (297). However, for severe disease caused by these viruses, high-risk hosts, or those who are hospitalized for respiratory symptoms, laboratory testing for a specific diagnosis may alter care.

The differential diagnosis of croup due to PIV infection includes upper airway obstruction due to foreign bodies or bacterial epiglottitis, an entity less commonly seen since the decline of *Haemophilus influenzae* type b infections.

LABORATORY DIAGNOSIS

Sample type and handling

The diagnosis of respiratory viral infections is critically dependent on the type and quality of the clinical specimen and proper handling of the specimen prior to laboratory studies (298). Adequate clinical specimens are needed to avoid false-negative diagnoses. The preferred specimen type for the diagnosis of RSV, PIV, and MPV in infants and young children is a nasal wash (299, 300), aspirate (301), or mid-turbinate swab. A nasal wash specimen is the classical sample used for viral diagnosis by culture and for obtaining samples for cytokines or antibodies. The amount of RSV present in nasal wash specimens from young children is very high, ranging from 10^3 to 10^8 PFU/ml (169, 302), or 10^3 to 10^{11} genome copies/ml by RT-PCR (mean, 7.6 $\log_{10}$)(303).

Nasopharyngeal swabs and mid-turbinate swabs are not as sensitive as nasal washes for viral diagnosis by culture or direct fluorescent antigen detection; however, they have good diagnostic yields with molecular detection methods (299, 304). Specimens from adults are less sensitive than those from children as a result of decreased viral load. Other clinical specimens which have proven useful for the detection of RSV, PIV, and MPV in patients of all ages include endotracheal aspirates collected from intubated patients (305), bronchoalveolar lavage (BAL) (81, 89), nasal mucosal epithelium collected by scraping (157), sputum (306), and lung tissue obtained by biopsy or at autopsy (81, 89, 166).

Virus isolation

Virus isolation in cell culture is slow and labor intensive and is being replaced by sensitive and rapid nucleic acid amplification-based molecular diagnostic methods. Clinical specimens should be transported on ice and inoculated onto cell culture lines within 4 hours of collection, and those that are not immediately processed should be flash frozen using alcohol and dry-ice baths (298). Cell lines such as HEp-2, A549, or Vero cells are best used for the isolation of RSV. Mixed cell cultures containing two or more cell lines have also been used with or without centrifugation to enhance respiratory virus detection (307). RSV is identified by a characteristic syncytial pattern formed by the infected cells (Fig. 12). For PIV isolation, primary or continuous monkey kidney cells (e.g., LLC-MK2) with trypsin added in the medium are equally sensitive (308). PIV-3 can produce a recognizable cytopathic effect with syncytium formation in continuous cell lines (309), but cytopathic effect is generally not detected with other PIV types. PIV 4 has a restricted host range in cell culture, growing mainly in LLC-MK2 cells without syncytium formation or typical cytopathic effect, except for cell rounding that progresses to destruction of cell monolayers (310). MPV has been cultivated in a number of cell types, most commonly LLC-MK2 (47). The addition of trypsin is required for efficient growth of MPV in culture. Cytopathic effect caused by MPV consists of cell rounding and syncytia (Fig. 13) and is generally not seen for several weeks after inoculation. MPV does not hemadsorb, but fluorescent antibody detection has been reported (311).

Antigen detection

Rapid diagnosis of RSV and PIVs using direct or indirect immunofluorescence (IF) methods became widely used with the advent of commercially available reagents (312–314) and has been adapted to detect MPV (311). Advantages of IF methods include the direct examination of clinical sample for epithelial cells (permitting specimen quality control), rapid results, and low cost. The IF method has become less widely used because it is less sensitive than molecular diagnostics, is not suitable for large-scale testing, and requires trained technicians and specialized equipment.

The availability of point-of-care tests (rapid antigen detection test) for the detection of RSV, PIV, and MPV antigen

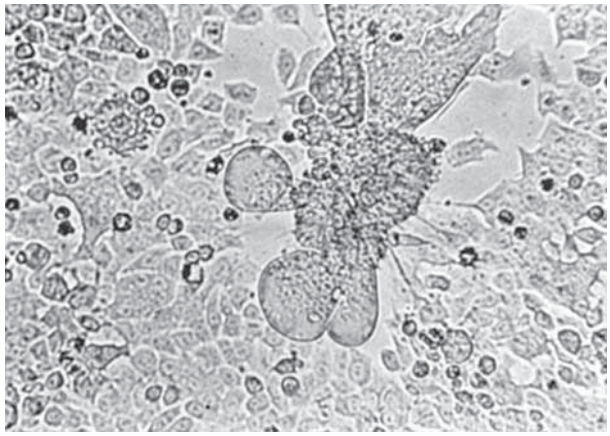

FIGURE 12 Cytopathic effect of RSV in cell culture on a continuous human epithelial cell line (HEp-2 cells) after 1 week of incubation. Large syncytial cells are noted, with ballooning and coalescence of RSV-infected cells.

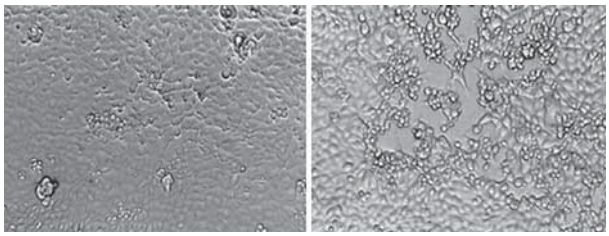

FIGURE 13 Cytopathic effect of MPV in cell culture on a continuous monkey kidney epithelial cell line (LLC-MK2 cells) after 14 days of incubation. Rounding up and detachment of cells are noted, with developing syncytia.

in clinical specimens provides a quick, reliable, and relatively inexpensive diagnostic test. Currently available point-of-care tests for RSV in pediatric specimens have sensitivities and specificities ranging from 80% to 95% (133, 315). Rapid antigen tests for PIV or MPV are not yet available in the United States. Antigen detection tests with nasopharyngeal specimens obtained from adult patients have much lower sensitivity, most likely because of the substantially lower viral titer (often <100 PFU of RSV/ml in immunocompetent adults) (169). In general, point-of-care tests do not require expensive equipment or highly skilled personnel, take only 15 to 20 minutes from start to finish, and are suitable for single specimens or batch testing of many specimens. Disadvantages include the lack of sensitivity compared to molecular diagnostic methods, absence of evaluation of the quality of the clinical sample, and potential false positives when samples with blood or mucus are tested. Point-of-care tests are often waived by the Clinical Laboratory Improvement Amendments, have been successfully used in clinics, at the bedside, and by non-laboratory personnel (314).

Nucleic Acid Detection

Molecular diagnostics, such as multiplex real-time PCR, have replaced cell culture, IF, and ELISA in many diagnostic laboratories because they can be automated, have high sensitivity and specificity, excellent quality control procedures, and detect a large battery of viral and bacterial pathogens rapidly in a single sample. The use of molecular diagnostics has significantly improved sensitivity compared to cell culture and IF, especially in adults (316). Reliable diagnosis of MPV currently depends on molecular techniques based on nucleic acid amplification assays. Several different RT-PCR methods are quite sensitive (303, 317, 318). Use of multiple simultaneous PCR reactions in clinical samples may be useful when PIVs and RSV are co-circulating (319). Numerous rapid multiplex PCR assays are now commercially available that can detect up to 18 or more viruses simultaneously (320, 321). More than a dozen single or multiplex molecular respiratory virus assays are FDA-cleared, with time to result ranging from less than 1 to 8 hours, but most (including all multiplexed assays) can currently be performed only in a clinical laboratory rather than at point-of-care. These assays are sensitive and specific and some can differentiate all four PIV subtypes (322). PCR sequencing assays have been applied to the molecular epidemiology of RSV, PIV, and MPV by analyzing a portion of the G or F protein gene (162, 323).

Antibody Assays

Acute- and convalescent-phase sera are generally required for the serologic diagnosis of RSV, PIV, or MPV. A 4-fold increase in antibody after at least 2 weeks, and preferably 3 to 4 weeks, or the appearance of specific IgM antibody is required for serologic confirmation of infection. Serologic assays have been useful in epidemiological and vaccine studies but less so clinically. Furthermore, young infants may not generate a reliable response to RSV or may have antibody responses obscured by maternal antibody. Immunocompromised patients or older persons who have had repeated infections may not demonstrate rises in antibody titer (298). Antibodies to RSV are sensitively measured by ELISA, neutralization, indirect IF, and plaque reduction (with or without complement enhancement) assays (305, 324, 325). RSV antibodies measured by the microneutralization test correlate best with protection from RSV disease in an animal model (326). Antibody to PIV in serum and respiratory secretions can be measured by complement fixation (CF), neutralization, or hemagglutination-inhibition (HI) techniques (309, 327). Careful interpretation of the CF or HI antibody results is required because heterologous cross-reactions are frequent among the paramyxovirus group, including mumps virus (309). Serologic evidence of previous MPV infection has been determined by measuring virus-neutralizing antibodies in plaque reduction assays (6) and by ELISA methods based on recombinant MPV proteins (328).

PREVENTION

The international health impact and economic burden attributed to RSV, PIV, and MPV is becoming more appreciated and contributes to the urgency of developing safe and effective vaccines against these pathogens (115, 329, 330). Progress has been slow (331), in part because of the serious adverse events following the use of the formalin-inactivated RSV (FI-RSV) vaccine in young children (332), early failures with live-attenuated virus vaccines, and the ineffective immune response elicited by primary RSV infection. The identification of serum neutralizing antibody as a correlate of protection against serious RSV lower respiratory tract disease has been an important advance in this field. Candidate vaccines against PIV and MPV have been tested in preclinical models, with limited human studies (38, 39, 333–337).

Management of nosocomial outbreaks

Nosocomial outbreaks characteristically occur from multiple introductions of community respiratory viral strains (338). For prevention of nosocomial transmission, contact isolation precautions are effective as long as compliance with the policy is maintained among personnel (339). The importance of isolation based on symptoms as opposed to viral-test positives has been shown, but prolonged shedding of respiratory viruses with even minimal symptoms may complicate efforts of infection control. Patients known or suspected to be infected with RSV, PIV, or MPV should be kept in contact isolation or cohorted together until symptoms have resolved and repeated sensitive diagnostic tests are negative (340).

Hospital personnel may play a role in the transmission of RSV to susceptible patients (162). Spread of RSV can be limited in these settings by aggregating infected and exposed individuals and adhering to strict hand-washing procedures, but hand washing is frequently neglected (341, 342). Use of gloves, masks, and goggles in the hospital setting will also limit spread (343). The use of masks and goggles can decrease nosocomial infections in hospitalized children and medical personnel by preventing viral infection of

TABLE 7 Control measures for the prevention of nosocomial RSV, PIV, and MPV infections

Institute contact isolation precautions (strict adherence).
Pay strict attention to good hand washing and/or use of gloves.
Wear a gown to prevent exposure of clothing to contaminated secretions.
Screen hospitalized patients for RSV, PIV, and MPV infections.
Cohort infected patients.
Exclude visitors with respiratory infections.
Prevent staff with respiratory illness from caring for susceptible patients.
Consider the use of eye-nose goggles when caring for subjects at high risk for severe viral infections.

personnel, who then transmit virus to susceptible individuals (343, 344). Strict compliance with glove and gown isolation precautions can also significantly reduce nosocomial RSV infections (339, 342). Such strict measures are appropriate in high-risk settings such as pediatric intensive care units or bone marrow transplant wards. Restriction of visitors, particularly young children, in hospital wards at high risk for RSV infection may be necessary during community epidemic periods. Continued compliance through the respiratory virus season by all members of the health care team is critical to any successful infection control policy. The Committee on Infectious Diseases of the American Academy of Pediatrics has provided guidelines for the prevention of nosocomial infections attributed to RSV and PIVs, as illustrated in Table 7 (345).

Prophylaxis

Passive immunoprophylaxis

A humanized monoclonal antibody specific for antigenic site II of the F protein of RSV, palivizumab (Synagis®), has been approved for use in high-risk children since 1998. It is currently the only FDA-approved monoclonal antibody for prophylaxis in infants and young children at increased risk of hospitalization with RSV infection. It was approved for preterm infants less than 35 weeks' gestational age, for infants with chronic lung disease of prematurity (CLD), and hemodynamically significant congenital heart disease (CHD). The administration of 15 mg of palivizumab per kg intramuscularly at 4-week intervals during the RSV season has been shown to be safe and effective in reducing hospitalization due to RSV by 55% in preterm children, children with CLD, and children with CHD (346, 347). Motavizumab, a more efficacious monoclonal antibody in the prevention of RSV in high-risk infants, was not approved by the FDA because of safety concerns (348, 349). Next-generation RSV F protein monoclonal antibodies that target antigenic sites on the pre-fusion F form with enhanced neutralizing activity or contain novel mutations in the Fc-domain (YTE) that extend half-life 3- to 4-fold, allowing a single dose per season, are undergoing clinical evaluation (Clinical Trials. Gov ID: NCT02325791 and NCT02290340). Other antibodies in development are broadly neutralizing monoclonal antibodies against both RSV and MPV (350, 351) and recombinant human polyclonal antibodies against viral infections including RSV (352).

The 2014 Guidelines issued by the American Academy of Pediatrics added additional restrictions for palivizumab prophylaxis among high-risk infants and young children, in part, driven by its high cost (353). The revised guidelines recommend that prophylaxis with palivizumab be considered for 1) preterm infants born before 29 weeks without CLD or CHD and who are younger than 12 months at the start of the RSV season; 2) preterm infants with CLD during the first year of life and whose CLD developed at less than 32 weeks' gestational age. A second year of prophylaxis may be considered for infants who continue to require medical support for CLD; and 3) infants 12 months old or younger with hemodynamically significant CHD during the first year of life, in particular, those with acyanotic heart disease who require medication for control of congestive heart failure or will require cardiac surgical procedures. Other high-risk groups to consider for prophylaxis are children during the first year of life with pulmonary abnormalities or neuromuscular disease and children younger than 24 months of age who are profoundly immunocompromised.

Passive immunoprophylaxis against PIV and MPV infection has not been studied.

Active immunization

There are currently no approved RSV, PIV, or MPV vaccines. In the 1960s, field studies were conducted with FI-RSV and PIV vaccines (332, 354). The FI-RSV vaccine produced by Pfizer was a crude RSV-monkey kidney cell harvest precipitated with a high concentration of alum and concentrated 100-fold. The PIV vaccine was prepared similarly. In all four studies, some children, usually less than 2 years of age, who received the FI-RSV vaccine experienced more severe respiratory disease, including some deaths, on subsequent infection with RSV. Enhanced disease was not observed in the PIV vaccinees. FI-RSV vaccinees had a nearly 8-fold-increased risk of pneumonia and a 16-fold-increased risk of hospitalization compared to the controls (175). The enhanced respiratory disease described in children less than 2 years of age appeared similar to naturally occurring disease in infants less than 6 months of age who were hospitalized with RSV bronchiolitis/pneumonia. Importantly, the FI-RSV vaccine produced by Merck was not associated with enhanced disease in young children (355, 356), suggesting that variables other than the RSV vaccine may have been required for the development of vaccine-enhanced disease. The pathogenesis of vaccine-enhanced disease remains poorly defined. Advances in the development of new RSV vaccines have been hampered by the inability to identify the immune mechanism responsible for vaccine-enhanced disease and a satisfactory animal model.

With the technical advancements in molecular virology and vector development, a greater understanding of the structure-function relationship of RSV proteins, and an expanding appreciation of RSV- related morbidity and mortality among children and older adults worldwide, there has been an explosion of candidate RSV vaccines at the preclinical and clinical phases of development (www.path.org/vaccinesresources). A broad array of vaccine formulations is being evaluated in animal models. These include live attenuated, whole-virus inactivated, virus like-particles, subunits, DNA or RNA, and vector-based RSV vaccines. Most of the inactivated, DNA, and vector vaccines are composed solely of or include as a major component the F gene or F protein in the vaccine formulation. This is because the F protein is relatively well conserved among the RSV/A and RSV/B genotypes, and it contains conserved antigenic sites that induce neutralizing antibodies. Two major conformational forms of the F protein occur in RSV, the prefusion and postfusion confirmations (217). Both the prefusion and postfusion conformational forms have unique

and shared antigenic sites. There is an ongoing debate as to which conformational form will lead to the better vaccine. Relevant to this discussion will be issues related to production, cost, antigen stability, safety, immunogenicity, and efficacy. A nanoparticle RSV-F vaccine currently leads the RSV vaccine field with two phase III trials started in the winter of 2015, one in older adults and a second in pregnant women. However, recently an RSV-F nanoparticle vaccine induced neutralizing antibodies and demonstrated 50% protection against RSV infection diagnosed by Western blot antibody assay in women of childbearing age (357). In a phase II trial in older adults, this RSV-F nanoparticle vaccine prevented 64% of moderate to severe RSV cases with 44% efficacy against symptomatic confirmed RSV cases (NCT02266628, ClinicalTrials.gov) (358). This is the first vaccine candidate to date that has been reported successful in preventing serologic and symptomatic RSV infection.

Live attenuated vaccines could potentially circumvent the issues related to the enhanced disease of FI-RSV vaccine and also take advantage of mucosal immunity. However, developing an attenuated, genetically stable, nontransmissible, and immunogenic live RSV vaccine for use in infants has proven difficult (331). Early attenuated cold-adapted temperature-sensitive phenotypes were found to require further attenuation by chemical mutagenesis (359, 360). Studies of genetically characterized live attenuated, temperature-sensitive RSV vaccines have been carried out in infants as young as 2 months of age (361). None of the infants and young children who received the live attenuated RSV vaccines developed enhanced disease on subsequent exposure to RSV (362). More recently, a live attenuated RSV vaccine with alterations in the M protein has shown enhanced immunogenicity in a small study conducted in seronegative children (363). Codon deoptimization is an alternative strategy being pursued to generate a stable, attenuated and immunogenic live attenuated RSV vaccine (364, 365).

Inactivated and live attenuated PIV vaccines have been studied. Two different strategies are currently under active development by the National Institute of Allergy and Infectious Diseases (NIAID) and industrial partners for PIV3 vaccines: candidates based on live-attenuated bovine parainfluenza type 3 (BPIV3) strains, and those based on a cold-adapted live attenuated PIV 3. BPIV3 vaccines have been tested in infants, children, and adults with good safety and immunogenicity (366–368). Evaluation of cold-adapted, live attenuated PIV type 3 vaccines in children has paralleled that of the live attenuated RSV vaccine (366). Newer live attenuated PIV vaccines have shown promise in clinical trials, with a cDNA-derived recombinant version of a PIV3 strain developed via repeated passages at low temperatures showing promise of safety and immunogenicity in young seronegative children (333). PIV subunit vaccines with proven efficacy in animal models have not been evaluated in clinical trials (369). The live attenuated bovine PIV-3 vaccine is also being studied as a vaccine vector (370, 371), using a chimeric construct expressing the PIV-3 F (fusion) and HN (hemagglutinin-neuraminidase) proteins and the RSV F protein. Such vaccines have been tested in seropositive and seronegative children and young infants (372).

Several approaches to MPV vaccines have been tested in animal models, including subunit proteins, chimeric virus vectors, T-cell epitope vaccines, virus like particles, and live attenuated virus (373). Recombinant F protein subunit vaccines for MPV have been demonstrated to be effective in rodents (39, 334). Peptide vaccines based on $CD8^+$ T-cell epitopes reduced viral titers in mice (374). Recombinant chimeric PIV and alphavirus-vectored vaccines that expressed MPV F, or avian/human MPV chimeric vaccines, were immunogenic and protective against challenge with MPV in rodents (337, 375, 376). Virus like particles induced neutralizing antibodies and T-cell responses and protected against challenge in mice (232, 335, 377). Live attenuated MPV vaccines are under development using reverse genetics approaches (378). These include gene deletion (G and/or SH), temperature-sensitive mutations, mutations in M2-1 or L, mutations in the N-glycosylation sites of F protein, and mutation of the integrin-binding RGD motif in the F protein (24, 38, 336, 379–381).

Maternal Immunization

RSV-specific serum neutralizing antibodies are efficiently transferred from the mother to the newborn (243, 382). High levels of neutralizing antibodies acquired transplacentally by the neonate protect against lower respiratory tract disease during the first few months of life. The decline of virus-specific immunity provided by maternal antibodies closely mirrors the half-life of IgG1, the principal IgG subclass antibody to RSV F and G glycoproteins that is transplacentally transferred in preterm and term neonates (382).

One strategy to protect infants younger than 6 months of age from RSV disease is to augment maternal antibody by administration of an RSV vaccine to the mother during pregnancy (383, 384). An RSV-F nanoparticle vaccine was shown to be well tolerated, safe for the mother and fetus, and immunogenic in a phase I randomized, placebo-controlled trial in pregnant women (NCT02247726, ClinicalTrials.gov) (385). A worldwide phase III trial of the RSV-F nanoparticle vaccine in pregnant women is ongoing (NCT02624947, ClinicalTrials.gov).

TREATMENT

Supportive Treatment

Previously healthy children and immunocompetent healthy adults infected with RSV generally require supportive treatment only. Antibiotic treatment is usually not necessary except for concomitant suspected bacterial AOM or sinusitis (272, 273). The potential hypoxemia, apnea, and poor oral intake resulting from infection in young infants require close medical management, and hospitalization may be required for children less than 1 year of age. Intravenous fluid replacement and oxygen therapy may be necessary. Secondary bacterial pneumonia is uncommon (272). Because the hypoxemia is related to unequal ventilation-to-perfusion ratios, infants will generally respond to inspired oxygen concentrations of 40% or greater (298). Corticosteroid therapy is not effective in the treatment of acute RSV and does not benefit pulmonary function during convalescence in young infants (386, 387). Thus, systemic or inhaled steroids are not recommended for treatment of RSV. Bronchodilator therapy is no longer recommended, based on studies that fail to show shortened hospital stay or improved outcomes (388). Supportive management in the care of lower respiratory tract disease due to RSV in the older child or high-risk adult may include oxygen therapy, close attention to fluid and electrolyte balance, and aerosolized bronchodilators. In patients with underlying pulmonary or heart disease, recovery from the effects of RSV infection may take weeks to months.

Treatment of croup, the most common clinical presentation of PIV infection, generally consists of reassurance of

the family and providing advice regarding the need for medical attention. Treatment of a child with croup in a mist tent has not been shown to be of benefit and is no longer recommended (389, 390). Systemic glucocorticoid therapy, including intramuscular dexamethasone, oral prednisolone, and nebulized budesonide is efficacious in mild to moderate and severe croup in the young child (391, 392). The decision to use and the route of administration—oral, injected, or aerosolized—should be based on the clinical assessment of the child, the ease of administration, cost, and duration of use. Epinephrine is used for symptomatic relief in patients with moderate to severe symptoms, but because the benefits of racemic epinephrine are short lived, such patients must be observed carefully after therapy to be sure that their clinical condition does not deteriorate after the effects of epinephrine have diminished. Children with airway obstruction or signs of hypoxia require admission to an intensive care setting for close monitoring and may benefit from treatment with intravenous dexamethasone; children with severe disease may require intubation. Antibiotic therapy is generally not beneficial except in cases of secondary bacterial infection, as suggested by persistent high fevers or purulent material noted at endotracheal intubation. Lower respiratory tract infections due to PIV in young children or immunocompromised hosts may require hospitalization and adjunct therapy, including intravenous fluids and oxygen support.

The majority of children infected with MPV can be managed at home with supportive care. For infants and children who require hospitalization, therapy is supportive, including supplementary oxygen and intravenous hydration. Bronchodilators and corticosteroids have been used empirically, but there are no controlled trials of these medications for MPV and no data to support or refute efficacy.

Management of Severe Disease

Infants with respiratory failure require mechanical ventilation and may require pressor support. Severe RSV disease in very young infants can result in life-threatening damage to the lungs and secondary end-organ failure affecting the cardiac, renal, and hepatic systems. Some success with the use of other ventilatory support modalities such as high-frequency ventilation, mixtures of gases such as helium and oxygen or nitrous oxide, or extracorporeal membrane oxygenation (ECMO) to permit healing of the lung has been reported. Prolonged supportive therapy with ECMO for days to several weeks may be required for RSV infection (393).

Upper respiratory tract disease due to RSV in immunocompromised hosts (81) may progress to severe lower respiratory tract disease requiring ventilatory support. Underlying immunosuppression, high inspired oxygen concentrations, and the accompanying barotrauma may result in pulmonary hemorrhage or acute respiratory distress syndrome or both. The presence of pneumonia and associated respiratory failure in severely immunocompromised patients generally culminates in multi-organ system failure, with mortality in intubated patients approaching 80% to 90% (81, 83, 166, 224). Supportive care in these immunocompromised patients includes fluid and nutritional support, as well as aggressive therapy of secondary fungal, bacterial, or viral infections. Antiviral therapy is frequently used (discussed below).

PIV infection with lower respiratory tract disease in immunosuppressed patients, particularly very young patients or those in the immediate post-transplantation period, may result in a similar clinical picture (166, 394), although the overall mortality does not appear to be as high as with RSV (230, 268). Supportive and management measures are similar to those used for RSV disease. Severe and fatal MPV infections have been reported in immunocompromised or other high-risk hosts, including very young children and the frail elderly (77, 78, 87–90, 153, 163, 257, 266). Mechanical ventilation and ECMO have been used to treat these patients.

Antiviral Treatment

Ribavirin, a synthetic guanosine nucleoside, has been licensed as an aerosol formulation for treatment of RSV respiratory disease in children since 1986 and for the treatment of RSV disease in mechanically ventilated patients since 1993. Ribavirin is the only approved drug for lower respiratory tract disease due to RSV (395), but concerns regarding efficacy in children, cost, and drug administration issues have resulted in minimal current use of the drug, except in immunocompromised patients (396). Ribavirin is administered by small-particle aerosol from a solution containing the drug at a standard concentration of 20 mg/ml sterile water via aerosol for approximately 20 hours per day. Aerosol administration results in high levels of ribavirin in the secretions, with levels exceeding 1,000 μM and little systemic absorption.

The duration of therapy in immunocompromised hosts with serious lower respiratory tract disease is generally greater than 5 days. Delayed antiviral treatment of RSV infections in markedly immunocompromised patients, such as bone marrow transplant recipients who receive antiviral therapy only after the initiation of mechanical ventilation, is not generally successful (264). Initiation of antiviral therapy at the stage of upper respiratory tract disease may decrease viral load and possibly reduce the risk of respiratory failure (397, 398). Intermittent therapy utilizing higher drug concentrations (60 mg of ribavirin/ml of water) administered over 2 hours three times daily to provide the same total amount of drug compared favorably with standard ribavirin therapy in one small clinical trial in children and in an uncontrolled trial in immunocompromised adults (398). This delivery method may improve patient access, improve compliance with therapy, and decrease environmental release of drug (399). A small randomized trial comparing short intermittent ribavirin in HCT patients with RSV upper respiratory tract disease demonstrated good tolerability and a trend of decreasing viral load over time compared to no treatment (400).

The potential environmental release of ribavirin has caused concern in hospital personnel because of the potential teratogenicity of ribavirin in the rodent model (401). Exposure is contraindicated in pregnant women because of its teratogenic potential. Administration of ribavirin via a ventilator, using a high-dose, short-duration method of drug delivery (399) or with a vacuum-exhausted treatment hood (402) results in minimal or no detectable ribavirin in the rooms of treated children.

Systemic antibody therapy (403), combined antibody therapy and ribavirin (264, 404), and aerosolized antibodies have been used for treating RSV disease (56). The combination of high-titer RSV immune globulin (RSVIG) and ribavirin has been associated with therapeutic success in uncontrolled studies in severely immunocompromised adults with RSV disease. Treatment studies with RSVIG (which is no longer available), palivizumab at 15 mg/kg, and motavizumab at 30 mk/kg have not proven efficacious in infants (405). Nanobodies, derived from the heavy-chain variable Ig domains that occur naturally in camels, administered by inhalation are being investigated for the treatment of RSV infection (406).

Two promising antiviral agents (GS-5806 and ALS-008176) have recently been shown to reduce viral load and clinical disease in an adult challenge model of RSV infection (407, 408). GS-5806 is a small molecule that inhibits virus entry by blocking viral fusion with cells in the respiratory epithelium, and ALS-008176 is an orally bioavailable prodrug of a nucleoside analogue that inhibits RSV replication by chain termination. Clinical treatment studies on these antiviral agents are ongoing.

There is no approved antiviral therapy for the treatment of PIV infections. Ribavirin inhibits PIV replication in cell culture and has been used for the treatment of lower respiratory tract disease in immunocompromised hosts (224). Case reports documenting decreased viral load and clinical improvement in children with severe combined immunodeficiency following multiple treatments with aerosolized ribavirin therapy have been published (99, 209). Ribavirin has also been utilized in bone marrow transplant recipients with upper and lower respiratory tract infection with PIV, without apparent benefit (224, 269). Intravenous ribavirin administered by intermittent dosing or constant infusion has been used in individual patients for the treatment of serious PIV infections (409), although retrospective studies of ribavirin in transplant recipients have not demonstrated convincing efficacy (270). A new potential antiviral therapy for PIV and other sialic acid-binding viruses targets the removal of lung epithelial sialic acid receptor for PIV, thereby preventing viral entry. A novel recombinant sialidase fusion inhibitor, DAS-181 (Ansun Biopharma, San Diego, CA), first developed as an antiviral agent for influenza, functions by cleaving sialic acids from the host cell surface, thereby inactivating the host cell receptor recognized by PIV (410). Successful use of this agent in pediatric and adult transplant recipients under compassionate use has been reported (411, 412), and a clinical trial in adult HCT recipients is ongoing.

Antiviral therapy for the treatment of severe MPV disease has not been studied in humans. One animal study suggested benefit with ribavirin and corticosteroid treatment of experimentally infected mice (413). Both ribavirin and polyclonal human immunoglobulin possessed *in vitro* virus inhibiting activity against MPV equivalent to their activity against RSV (414). A number of agents have shown efficacy *in vitro* or in animal models, including NMSO3, a sulfated sialyl lipid (415, 416); monoclonal antibodies (351, 417, 418); fusion inhibitor peptides (419, 420); and short interfering RNA (421, 422). There are numerous case reports of ribavirin and polyclonal intravenous immunoglobulin (IVIG) in severely immunocompromised patients (163, 423), but no controlled trails have been published for these interventions.

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Measles

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38

Measles is a highly contagious disease caused by infection with measles virus (MeV), and it has caused millions of deaths since its spread within human populations thousands of years ago. Disease begins with fever, cough, coryza, and conjunctivitis followed by the appearance of a characteristic maculopapular rash. Genetically, MeV is most closely related to rinderpest virus, a pathogen of cattle that was recently eradicated. MeV was originally a zoonotic infection that adapted to humans 5,000 to 10,000 years ago when populations achieved sufficient size in Middle Eastern river valley civilizations to maintain a continuous chain of transmission among susceptible individuals. Subsequent introduction of MeV into naive populations resulted in high mortality. Millions died as a result of European exploration of the New World, largely due to the introduction of diseases such as smallpox and measles into native Amerindian populations (1).

Abu Becr, an Arab physician also known as Rhazes, first distinguished smallpox from measles in a 9th century treatise on the two diseases. Peter Panum, a Danish physician sent to the Faroe Islands in 1846 during a large measles epidemic, first described several of the basic epidemiological features of measles (2), including the highly contagious nature of MeV infection, the 14-day incubation period, and the lifelong immunity following infection. Following the practice of variolation to protect individuals from smallpox, Francis Home, a Scottish physician working in the 18th century, attempted to immunize children by inoculating their scarified skin with blood taken from infected individuals shortly after the rash appeared (3). Joseph Goldberger and John F. Anderson reproduced the disease in monkeys inoculated with filtered respiratory tract secretions from patients with measles, thus demonstrating that a virus was the cause of measles. MeV first was isolated from the blood and propagated in cell culture in 1954 by John Enders and Thomas Peebles, laying the foundation for the development of attenuated measles vaccines (4).

VIROLOGY

Classification

MeV is a member of the genus *Morbillivirus* within the family *Paramyxoviridae*. Other members of the genus include canine distemper virus, which affects dogs and other mammalian carnivores; rinderpest virus, which affects domestic cattle and swine; peste des petits ruminants virus, which affects sheep and goats; phocine distemper virus, which causes epizootic disease in seals; and porpoise and dolphin morbilliviruses, which cause epizootic disease in porpoises and dolphins, respectively. Morbilliviruses differ from other paramyxoviruses in lacking neuraminidase activity and in forming intranuclear inclusion bodies as a distinctive feature of their cytopathology.

Serotypes

Only one serotype of MeV exists, and recovery from measles confers lifelong immunity to reinfection. Although antigenic changes have been detected in the hemagglutinin surface protein, these variations have not reduced the protective immunity induced by wild-type MeV infection or measles vaccines. MeV remains a monotypic virus, likely because of functional constraints on the amino acid sequence and tertiary structure of the MeV surface proteins (5–7).

Genetic and Antigenic Variation

Despite the high degree of genetic variation expected of a single-stranded RNA virus, analysis of hemagglutinin (H), fusion (F), nucleoprotein (N), and phosphoprotein (P) gene sequences have shown that MeVs isolated during the 1950s and 1960s, including vaccine strains, were remarkably homogeneous and differed in sequence by no more than 0.5% to 0.6% at the nucleotide level. Sequence analysis of more recent wild-type MeVs has demonstrated some genetic variability relative to vaccine strains and these older wild-type viruses, particularly in the N and H proteins. One of the most variable regions of the MeV genome is the 450-nucleotide sequence at the carboxy-terminus of the N protein, with up to 12% variability between wild-type viruses that has been useful for genotyping. The World Health Organization (WHO) currently recognizes eight clades of MeV (designated A through H) and 24 genotypes. New genotypes may be identified with improved surveillance and molecular characterization. As measles control efforts intensify, molecular surveillance of circulating MeV strains can be used to document interruption of transmission and to identify the source and transmission pathways of MeV outbreaks (8, 9).

These genetic changes are accompanied by minor antigenic differences in the corresponding H, N, and matrix (M) proteins of some wild-type MeV isolates (10). Serum

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specimens from vaccinated persons and from persons naturally infected during the 1950s and 1960s have comparable neutralization activity against vaccine viruses and recently isolated wild-type MeVs, although sera from persons infected in the 1990s have higher neutralization titers to the homologous wild-type virus (11). As an example of how genetic changes have failed to alter important antigenic epitopes, MeV isolates from a genotype circulating in the People's Republic of China during 1993 and 1994 differed from other wild-type viruses by as much as 6.9% in the H gene and 7% in the N gene. However, they did not differ significantly from other wild-type viruses in their anti-H monoclonal antibody binding patterns, and they were neutralized by human post-vaccination antiserum (12). Thus, neutralizing epitopes on the H protein are highly conserved among MeVs. Many centuries of selective pressure exerted by naturally acquired immunity and more recently by vaccine-induced immunity have not resulted in the selection of new antigenic types.

Composition of the Virus

Virion Morphology

Measles virions are spherical, enveloped particles with a helical nucleocapsid and are morphologically indistinguishable from the virions of other paramyxoviruses (Fig. 1). Virion diameter averages 150 nm and varies between 100 and 250 nm. The envelope, a lipid bilayer derived from the plasma membrane of the infected host cell, carries surface projections composed of two transmembrane glycoproteins, the H and F proteins. On the inner surface of the envelope is the M protein, which interacts with the nucleocapsid and cytoplasmic tails of the H and F transmembrane glycoproteins to play a key role in virus maturation (Fig. 2). The helical nucleocapsid is packed within the envelope in the form of a symmetrical coil consisting of about 2,500 copies of the N protein encapsidating the genomic RNA, together with small amounts of the P and the large polymerase (L) proteins.

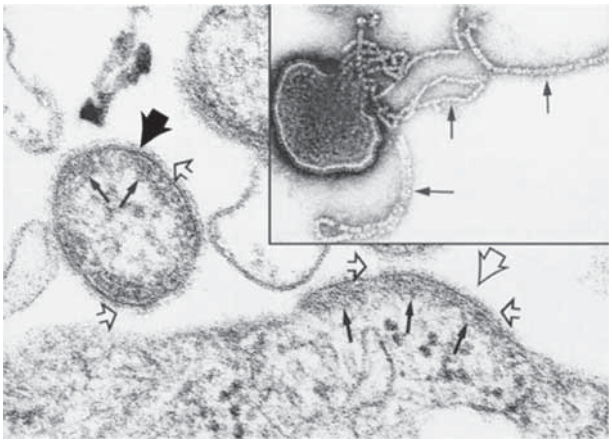

FIGURE 1 Measles virus (MeV) in cell culture. An extracellular virion (large solid arrow) is coated with glycoprotein spikes (small open arrows) with the viral nucleocapsid (small solid arrows) positioned beneath the envelope. An infected cell has a region on the membrane (large open arrow) with viral glycoprotein spikes and subjacent viral nucleocapsids that is a site of MeV maturation and budding. Free paramyxovirus nucleocapsids (small solid arrows) from a disrupted virion are shown in the inset. (Courtesy of Cynthia Goldsmith, William Bellini, and Erskine Palmer of the Centers for Disease Control and Prevention, Atlanta, GA.) [Adapted from Oxman, 2nd edition of *Clinical Virology*.]

Genome

The MeV genome consists of linear, single-stranded, nonsegmented RNA of negative polarity and contains about 16,000 nucleotides (Fig. 2), although the number of nucleotides can vary between virus strains (based on date and location of the measles virus isolate or sequence) and even between viruses of the same strain. At the 3′ end of the genome, a 53-nucleotide leader sequence shows a high degree of complementarity to the extragenic 40 nucleotide trailer sequence at the 5′ end, allowing the formation of a stable panhandle structure. The leader contains sequences that promote encapsidation of the nascent RNA by N protein and binding sites for the viral RNA polymerase. After the leader sequence are six consecutive non-overlapping genes that encode the N, P, M, F, H, and L proteins (Fig. 2). The P gene encodes two additional nonstructural proteins, C and V. The intergenic regions consist of a single trinucleotide, GAA, with a single variation between the H and L genes. In addition, there is an untranslated GC-rich region of about 1,000 nucleotides at the M-F gene boundary that spans the 5′ end of the M gene and the 3′ end of the F gene.

Structural and Regulatory Proteins

Six MeV gene products are structural proteins (Table 1). Three proteins, N, P, and L, are complexed with viral RNA to form the nucleocapsid, and three other proteins, M, H, and F, participate in the formation of the virus envelope (13). There is a transcriptional gradient from N to L for the mRNAs that encode these proteins, which determines their relative abundance. The N mRNA is transcribed first from the genome and thus is the most abundant. When expressed alone, N is an insoluble protein that migrates to the nucleus and self-assembles into helical nucleocapsid-like structures. When coexpressed with P in MeV-infected cells, N is retained in the cytoplasm as a soluble N-P complex. N binds both to RNA and to P, and it is required for transcription and replication. The N protein surrounds genomic and antigenomic RNAs that possess the leader sequence to form ribonucleocapsid structures that form the templates for both mRNA transcription and RNA replication. The conserved N-terminal portion of the N is required for self-assembly into nucleocapsids and for RNA binding (14). The variable C-terminal 125 residues form an intrinsically disordered region structurally similar to the acidic activation domains of cellular transcription factors (15). Sequence differences in the variable C-terminus of N provide the basis for the identification of different MeV genotypes.

The P protein is a polymerase cofactor activated by phosphorylation that forms tetramers and links L to N to form the replicase complex. The P protein modulates the assembly of functional MeV nucleocapsids after binding to individual molecules of N protein in the cytoplasm to form a soluble N-P complex that is required for RNA encapsidation. P also binds to the L protein to form an L-P complex involved in mRNA transcription and genome replication. The P gene of MeV, as in many members of the *Paramyxoviridae* family, encodes nonstructural proteins in addition to P. C is a basic protein translated using an alternate initiator methionine codon in an overlapping reading frame. V shares the P protein initiator methionine and the amino terminal 231 amino acids, but a non-templated guanosine residue is added through RNA editing, which shifts

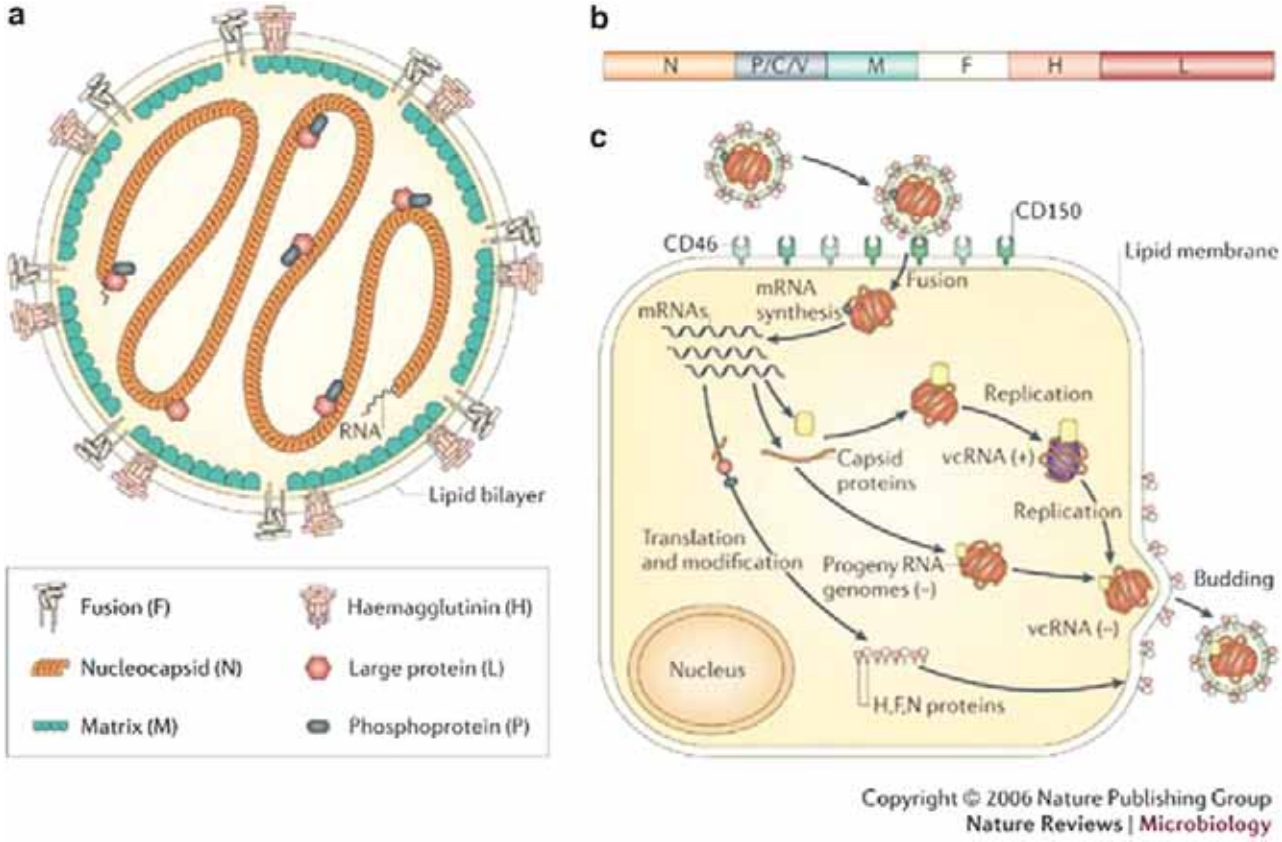

FIGURE 2 MeV structure, genome, and replication cycle. (a) MeV is a spherical, nonsegmented, single-stranded, negative-sense RNA virus. Of the six structural proteins, the phosphoprotein (P), large protein (L), and nucleoprotein (N) form the nucleocapsid that encloses the viral RNA. The hemagglutinin protein (H), fusion protein (F), and matrix protein (M), together with lipids from the host cell membrane, form the viral envelope. (b) The MeV RNA genome is comprised of approximately 16,000 nucleotides encoding eight proteins, two of which (V and C) are nonstructural proteins alternatively translated from the P gene. (c) The H protein interacts with F to mediate attachment and fusion of the viral envelope with the host cell membrane through specific receptors (CD46 and CD150) enabling viral entry into the cell. Remaining MeV proteins are involved in viral replication. The P protein regulates transcription, replication, and assembly of nucleocapsids. The M protein is critical for viral assembly. (From reference 127 with permission of the publisher.) Source: Moss WJ, Griffin DE. Global measles elimination. *Nat Rev Microbiol* 2006;4:900–908.

the reading frame to produce a different cysteine-rich C-terminus that has zinc-binding properties. Neither C nor V is necessary for MeV replication *in vitro* but both interact with cellular proteins to regulate the innate response to infection (16–18).

The L protein interacts with the P protein to form the MeV RNA-dependent RNA polymerase. Although P is required for polymerase activity, the L protein, which contains six regions that are highly conserved among the RNA polymerases of negative-stranded RNA viruses, has the catalytic activity for RNA synthesis.

H is the receptor-binding protein and an important determinant of cellular tropism. It is a type II transmembrane glycoprotein present on the surface of infected cells and virions as a disulfide-linked homodimer that self-associates to form tetramers (19). H has a 34 amino acid cytoplasmic tail preceding a single hydrophobic transmembrane region and a large C-terminal ectodomain with a propeller-like structure and 13 strongly conserved cysteines. The primary function of H is to bind to MeV receptors on the surface of host cells (19–21). The H protein is also responsible for the ability of measles virions to agglutinate simian erythrocytes by binding to a simian homologue of human CD46. Antibodies that block hemagglutination generally also neutralize virus infectivity. A second essential function of H is to interact with the F protein to mediate fusion of the virion envelope with the host cell membrane for delivery of the ribonucleocapsid into the cell (19, 22).

F is a highly conserved type I transmembrane glycoprotein, synthesized as an inactive precursor F_0 that is processed to the active disulfide-linked F_1 and F_2 that cooperate with H for fusion and entry. After synthesis and glycosylation in the endoplasmic reticulum, F_0 is transported to the Golgi where it is cleaved by furin into F_1 and F_2 (Table 1). The F_1 subunit, derived from the carboxy-terminus of F_0, is anchored in the viral envelope and has a cytoplasmic tail, the terminal 14 amino acids of which are highly conserved among morbilliviruses. At the amino terminus of F_1 there is a 25 amino acid hydrophobic region, the fusion peptide, which interacts with the host cell membrane to induce fusion and is highly conserved among *Paramyxovirus* fusion proteins. Fully processed active $F_{1,2}$ proteins are present as trimers on the surface of infected cells and virions. Although the function of F is to fuse the viral envelope with the host cell membrane, F alone is not sufficient to induce fusion and coexpression of H is required. The interaction of H with the

TABLE 1 Major structural and regulatory proteins of MeV

Protein	Amino acids	Location within infected cell	Location within virion	Function and special features
Nucleoprotein (N)	525	Cytoplasm	Nucleocapsid	Most abundant of viral proteins. Closely associated with full-length and positive sense RNA to form nucleocapsids.
Phosphoprotein (P)	507	Cytoplasm	Nucleocapsid	More abundant in infected cells than in virions. Regulation of transcription and replication. Complex formation with N and L.
C	186	Cytoplasm and nucleus	Not present	Read from the same transcript as P, but in different reading frame. Regulates transcription and sensitivity to antiviral effects of interferon α/β.
V	298	Cytoplasm	Not present	Read from an "edited" transcript of P gene. Regulates transcription and sensitivity to antiviral effects of interferon α/β.
Matrix (M)	335	Inner leaflet of plasma membrane; cytoplasm in persistent infections	Inner surface of membrane	Virion assembly and budding.
Fusion (F)	553	Endoplasmic reticulum, Golgi and plasma membrane	Type I transmembrane surface protein	Glycosylated; membrane fusion, virus entry, and hemolysis activity in conjunction with H; active form cleaved from F_0 to F_1 and F_2.
Hemagglutinin (H)	617	Endoplasmic reticulum, Golgi and plasma membrane	Type II transmembrane surface protein	Glycosylated. Receptor binding. Hemagglutination activity. Disulfide-linked dimer.
Large (L)	2213	Cytoplasm	Nucleocapsid	Catalytic component of viral polymerase. Least abundant protein. RNA transcription and replication. Complex formed with P.

Adapted from reference 26.

cellular receptor induces a conformational change in F that brings the fusion peptide region of the F_1 subunit trimer into a position to initiate fusion (22). Synthetic peptide analogs of the fusion peptide inhibit both cell fusion and virus penetration, but they do not prevent virus attachment.

M is a basic protein with several conserved hydrophilic domains and is the second most abundant viral protein. It forms a continuous layer on the inner surface of the envelope and interacts with progeny nucleocapsids and with the cytoplasmic tails of F and H to mediate virion maturation. Binding of M to nucleocapsids inhibits transcription of MeV mRNA (23, 24).

Biology

Receptors

Three cellular receptors for MeV have been identified: membrane cofactor protein or CD46 (25, 26), signaling lymphocyte activation molecule (SLAM) or CD150 (27), and poliovirus receptor-like protein-4 or nectin-4 (28, 29). CD46 is a widely distributed human complement regulatory protein expressed on all nucleated cells. It acts as a cofactor for the proteolytic inactivation of C3b/C4b by factor I, but also induces proliferation and differentiation of regulatory T cells (30). SLAM is an important costimulatory molecule expressed on activated cells of the immune system (31). The cytoplasmic domain has tyrosines and SH-2 domain-binding regions that constitute an immunoreceptor tyrosine-based switch motif that binds small SH-2 domain adaptor proteins important for cell signaling. Nectin-4 is an adherens junction protein of epithelial cells. Both vaccine and wild-type strains of MeV can use SLAM and nectin-4 as receptors. Vaccine strains tend to use CD46 efficiently, while wild-type strains do not (32). The receptor binding regions for CD46 and SLAM on H are contiguous or overlapping and most H proteins can bind both receptors, but affinity and efficiency of entry differ (19, 21). In general, binding affinity for SLAM is higher than for CD46, and differences in the efficiency of receptor usage may involve interactions with F.

MeV probably uses additional receptors. The distributions of the known receptors in tissues do not account for the tropism and sites of MeV replication in acute infections, in which endothelial cells as well as immune system and epithelial cells are infected (33), or in chronic infections, in which cells of the central nervous system (CNS) are important targets for infection (34). Receptors used by attenuated vaccine strains adapted to growth in cells from nonsusceptible hosts, such as chickens, probably represent an additional category of MeV receptors that have not been identified.

Replication

Infection is initiated when the MeV H on the virion envelope attaches to the receptor. The fusion peptide at the amino terminus of the F_1 subunit, which is physically associated with H, undergoes a conformational change that results in fusion of the envelope with the cell membrane and delivery of the viral nucleocapsid into the cell cytoplasm (22). The virion RNA-dependent RNA polymerase (L) is then activated and begins transcribing monocistronic mRNAs from the nucleocapsid template.

Transcription occurs sequentially following the gene order. The polymerase terminates synthesis at the end of each gene following polyadenylation and reinitiates at the

consensus sequence of the next gene, without transcribing the intergenic nucleotides. The viral mRNAs are capped and polyadenylated. The poly-A tails are synthesized by a slippage or stuttering mechanism in which the polymerase reiteratively copies the sequence of four to seven Us at the end of the gene. The polymerase sometimes fails to reinitiate and detaches from the nucleocapsid, resulting in the mRNA abundance gradient (35).

The envelope glycoproteins H and F are synthesized and glycosylated in the endoplasmic reticulum, further processed in the Golgi, and transported to the cytoplasmic membrane as oligomers to form the H and F peplomers. The other MeV proteins accumulate in the cytoplasm. The availability of N appears to regulate the transition from mRNA transcription to viral RNA replication because the synthesis of genome-length, positive- and negative-sense RNAs is coupled to their concomitant encapsidation by N. To initiate encapsidation, the RNA polymerase catalyzes the sequence-specific binding of N from soluble N-P complexes to nascent leader RNA, releasing P protein. The continuing encapsidation by N, which is coupled to further RNA synthesis, masks the consensus signals at each gene boundary, preventing termination and RNA processing and yielding the full-length nucleocapsid. Positive- and negative-sense RNAs are encapsidated, but the majority of the nucleocapsids contain negative-sense viral RNA. A small number of P-L polymerase complexes associate with each of the nucleocapsids.

Measles virions bud from the plasma membrane, with progeny nucleocapsids attaching to growing actin filaments. Growth of the actin filament transports the nucleocapsid from the cytoplasm to the plasma membrane, initiating the budding process. M binds to newly synthesized nucleocapsids and also associates with the inner surface of the cytoplasmic membrane, with the cytoplasmic tails of H and F. Association with other M proteins excludes cellular membrane proteins from patches of cytoplasmic membrane destined for virion budding (36, 37).

Host Range

Because of the specificity of the host receptors and high infectivity of MeV, humans are the only natural host for the virus. Nonhuman primates can be infected experimentally and develop an illness similar to measles in humans, thus serving as models for measles pathogenesis and the evaluation of vaccines. However, native populations of nonhuman primates are not of sufficient size to maintain MeV transmission in the wild. Rodent-adapted strains of MeV have been developed by repeated intracerebral passage of virus in newborn animals. Although these strains do not produce an acute disease resembling measles in humans, they are used as models of MeV pathogenesis in the nervous system.

Growth in Cell Culture

Primary cultures of human and monkey kidney cells have traditionally been used for isolating MeV. Problems with supply and the potential contamination of primary monkey cells with simian viruses led to the use of continuous monkey kidney cell lines (e.g., Vero cells) for propagation of tissue culture-adapted strains of MeV. Isolation and propagation of wild-type strains of MeV are most efficient in cells that express SLAM. Most commonly used are the Epstein-Barr virus-transformed marmoset B lymphocyte cell line B95-8 and Vero cells engineered to express human SLAM (38, 39).

The incorporation of MeV H and F proteins into the plasma membranes of infected cells causes them to fuse with adjacent infected and uninfected cells. Consequently, replication of wild-type MeV in permissive cells results in characteristic cytopathic effects that include the formation of multinucleated giant cells as well as the production of eosinophilic intranuclear and intracytoplasmic inclusion bodies. Eosinophilic Cowdry type A intranuclear inclusion bodies are characteristic of morbillivirus infection. The intranuclear inclusion bodies are composed of helical nucleocapsids that appear smooth by electron microscopy and contain only the N protein. In contrast, intracytoplasmic inclusions are composed of helical nucleocapsids that appear "fuzzy" by electron microscopy and contain P and M proteins in addition to N.

Inactivation of MeV by Physical and Chemical Agents

MeV is inactivated by detergents and by lipid solvents such as ether or acetone. MeV is acid labile, losing infectivity below pH 4.5, and it is also inactivated by proteolytic enzymes, drying on surfaces, and exposure to sunlight. MeV is also thermolabile, with a half-life of 2 hours at 37°C, and it is completely inactivated in 30 minutes at 56°C. MeV may retain infectivity for a week at 0°C, and it can be stored for long periods at −70°C. MeV can be freeze-dried, and lyophilized virus is stable for prolonged periods at refrigerator temperatures (0°C to 8°C), a characteristic important for the storage and transportation of attenuated measles vaccines.

EPIDEMIOLOGY

Measles continues to be an important global cause of child mortality. Deaths from measles are due largely to an increased susceptibility to secondary bacterial and viral infections, attributed to a prolonged state of immune suppression. The disease burden caused by measles has decreased substantially over the past decades because of a number of factors. Measles mortality declined in developed countries in association with economic development, improved nutritional status, and supportive care, particularly antibiotic therapy for secondary bacterial pneumonia. The introduction of measles vaccines beginning in the 1960s led to substantial reductions in measles incidence, morbidity, and mortality in both developed and developing countries. Overall global measles mortality in 2014 was estimated to be 114,900 deaths (uncertainty bounds 53,700 and 330,000 deaths), a 79% reduction since 2000 (40). Despite this enormous progress, measles remains a leading vaccine-preventable cause of childhood mortality and continues to cause outbreaks in communities with low vaccination coverage.

Geographic Distribution

Measles can occur anywhere in the world, wherever MeV is circulating or introduced into susceptible populations. Small isolated populations, such as island populations, cannot sustain MeV transmission because of exhaustion of susceptible individuals and require the importation of MeV for outbreaks to occur. In the United States, the number of measles cases declined 99.9% by 2006 following the introduction of measles vaccine, but outbreaks in unvaccinated, susceptible populations continue to occur through importation of MeV (41–43). Persons in the United States who do not receive measles vaccine because of philosophical or

religious exemption are at particular risk of measles following importations (41, 44).

Incidence and Prevalence of Infection

MeV is one of the most highly contagious, directly transmitted pathogens and outbreaks can occur in populations in which less than 5% to 10% of persons are susceptible. Chains of transmission commonly occur among household contacts, school-age children, and health care workers. The contagiousness of MeV is best expressed by the basic reproductive number R_0, which represents the average number of secondary cases that arise if an infectious agent is introduced into a completely susceptible population. In the 1951 measles epidemic in Greenland, the index case attended a community dance, which resulted in an R_0 of 200 (45), an unusually high R_0 that demonstrates the potential infectiousness of MeV. In more typical settings, the estimated R_o for MeV is 12 to 18, compared to only five to seven for smallpox and rubella viruses. The high infectivity of MeV implies that a high level of population immunity is required to interrupt MeV transmission.

There are no latent or epidemiologically significant persistent MeV infections and no animal reservoirs. Thus, MeV can only be maintained in human populations by an unbroken chain of acute infections and transmission events, requiring a continuous supply of susceptible individuals. Newborns become susceptible to measles when passively acquired, transplacental maternal antibodies are catabolized, providing the main source of new susceptible individuals. For births to provide a sufficient number of susceptible persons to maintain MeV transmission, a critical community size of 300,000 to 500,000 persons with 5,000 to 10,000 births per year is required (46). In smaller populations, episodic outbreaks are dependent upon the importation of MeV by infectious individuals.

Prior to the introduction of measles vaccine, more than 130 million cases of measles and 7 to 8 million deaths occurred globally each year, and almost everyone was infected during childhood or adolescence. In the United States, the prevalence of antibodies to MeV in 18-year-olds exceeded 98%, and the incidence of measles, as in almost every other country, was equal to the number of surviving newborns. The widespread use of attenuated measles vaccines significantly reduced the incidence of measles and lowered measles morbidity and mortality.

Epidemic Patterns

When endemic, measles has a typical temporal pattern of incidence characterized by yearly seasonal epidemics superimposed upon longer epidemic cycles of 2 to 5 years or more. In temperate climates, annual measles outbreaks typically occur in the late winter and early spring. These annual outbreaks are likely the result of social networks facilitating transmission (e.g., congregation of children at school) and environmental factors (e.g., low humidity and ambient temperatures) favoring the infectivity and transmission of MeV (47). Measles cases continue to occur during the interepidemic period in large populations but at low incidence. The longer cycles occurring every several years result from the accumulation of susceptible persons over successive birth cohorts and the subsequent decline in the number of susceptible persons following an outbreak. The interval between epidemics is shorter in populations with high birth rates because the number of susceptible individuals reaches the epidemic threshold more quickly. Measles vaccination programs that achieve coverage rates in excess of 80% extend the interepidemic period to 4 to 8 years by reducing the number of susceptible individuals.

Age-Specific Attack Rates

Secondary attack rates in susceptible household and institutional contacts generally exceed 90%. The average age of MeV infection depends upon the rate of contact with infected persons, the rate of decline of protective maternal antibodies, and the vaccine coverage rate. Infants in the first few months of life are protected by passively acquired maternal antibodies, and measles is rare in this age group. In densely populated urban settings with low vaccination coverage rates, measles is a disease of young children. The cumulative incidence can reach 50% by 1 year of age, with a significant proportion of children acquiring MeV infection before 9 months, the age of routine vaccination in many countries. As measles vaccine coverage increases, or population density decreases, the age distribution shifts toward older children. In such situations, measles cases predominate in school-age children. Infants and younger children, although susceptible if not protected by immunization, are not exposed to MeV at a rate sufficient to cause a large disease burden in this age group. As vaccination coverage increases further, the age distribution of cases may be shifted into adolescence and young adulthood, as seen in measles outbreaks in the Americas (48, 49), necessitating targeted measles vaccination programs for these older age groups. This shift in measles cases to older age groups has recently been observed in many countries as a consequence of moderately high measles vaccine coverage (e.g., 60% to 80%), allowing susceptible individuals to age into adolescence and young adulthood without being exposed to either wild-type MeV or measles vaccine.

As noted above, young infants in the first months of life are protected against measles by maternally acquired IgG antibodies. An active transport mechanism in the placenta is responsible for the transfer of IgG antibodies from the maternal circulation to the fetus starting at about 28 weeks of gestation and continuing until birth. Three factors determine the degree and duration of protection in the newborn: (i) the level of maternal anti-MeV antibodies; (ii) the efficiency of placental transfer; and (iii) the rate of catabolism in the child. Although providing passive immunity to young infants, maternally acquired antibodies can interfere with the immune responses to the attenuated measles vaccine by inhibiting replication of vaccine virus (50, 51). In general, maternally acquired antibodies are no longer present in the majority of children by 9 months of age (52), the time of routine measles vaccination in many countries. Women with vaccine-induced immunity tend to have lower anti-MeV antibody levels than women with naturally acquired immunity, and their children may be susceptible to measles at an earlier age. The half-life of anti-MeV antibodies has been estimated to be 48 days in the United States and Finland but is shorter in some developing countries. Infants born to human immunodeficiency virus (HIV)-infected women may have lower levels of protective maternal antibodies independent of their own HIV infection status and may thus be susceptible to measles at a younger age (53).

Subclinical Infection

MeV infections in nonimmune individuals are almost always symptomatic. Subclinical measles is defined as a 4-fold rise in MeV-specific IgG antibodies following exposure to wild-type MeV in an asymptomatic individual. Subclinical infection

may be important in boosting protective antibody levels in children with waning immunity (54). Whether partially immune individuals with subclinical infection can sustain MeV transmission is unknown. However, MeV has been isolated from a naturally immune, asymptomatically reinfected individual (55), and acquisition from a person with subclinical infection was implicated in at least one investigation.

Wild-type MeV infection induces lifelong immunity to disease, and reinfection is not required to maintain this protective immunity. On re-exposure, immune individuals may be reinfected and support limited virus replication, as evidenced by increases in their preexisting levels of humoral and cellular immunity to MeV. Such reinfections are almost always asymptomatic and rarely result in transmission of MeV to susceptible contacts (as with subclinical infections).

Seasonality

In temperate climates the incidence of measles peaks in late winter and early spring and reaches a nadir in late summer and early autumn. These differences are less pronounced in tropical climates. Where widespread immunization has reduced MeV transmission, the temporal distribution of cases is determined by importations of MeV and outbreaks may occur at any time.

Transmission

MeV is transmitted primarily by respiratory droplets over short distances and less commonly by small particle aerosols that remain suspended in the air for long periods of time or by direct contact with infected secretions. The symptoms induced during the prodrome, particularly sneezing and coughing, enhance transmission. Airborne transmission appears to be important in certain settings, including schools, physicians' offices, hospitals, and enclosed public gathering places (56). Direct contact with infected secretions can transmit MeV, but the virus does not survive long on fomites and is inactivated by heat and ultraviolet radiation. Transmission across the placenta can occur when measles occurs during pregnancy, but congenital measles is uncommon.

Duration of Infectiousness

Persons with measles are infectious for several days before and after the onset of rash, when levels of MeV in blood and body fluids are highest and when the symptoms of cough, coryza, and sneezing are most severe. The fact that MeV is contagious before the onset of recognizable disease hinders the effectiveness of quarantine measures. MeV can be isolated from the urine as late as 1 week after rash onset. MeV shedding is prolonged in those with impaired cell-mediated immunity. Giant cells were detected in nasal secretions up to 28 days after the onset of rash in malnourished Kenyan children with severe measles (57), and MeV antigen was detected up to 13 days after rash onset in malnourished Nigerian children (58). Prolonged presence of MeV RNA has been associated with HIV infection (59, 60) and congenital measles (61). However, whether detection of MeV by these methods indicates prolonged contagiousness is unclear.

Risk Factors for Transmission

The risk of MeV transmission is increased by more frequent, prolonged, and intimate contact between susceptible persons and infectious cases. The risk of transmission is substantially greater if contact occurs when the index case is in the late prodromal stage with more pronounced coryza, cough, and sneezing rather than later when these symptoms have abated. Patterns of air circulation may determine the risk of transmission when airborne transmission is involved. In daycare centers and schools, the risk of transmission often exceeds 50% to susceptible contacts.

Nosocomial Transmission

Medical settings are well-recognized sites of MeV transmission. Patients may present to health care facilities during the prodrome when the diagnosis is not obvious, although the patient is infectious and likely to infect susceptible contacts. Health care workers can acquire measles from infected patients and transmit MeV to others. Nosocomial transmission can be reduced by maintaining a high index of clinical suspicion and using airborne isolation precautions when measles is suspected, and it can be prevented by administering measles vaccine before exposure to susceptible patients and health care workers, as well as documenting immunity to measles (i.e., receipt of two doses of measles vaccine or detection of antibodies to MeV) in health care workers. If a health care provider without evidence of immunity is exposed to measles, measles vaccine should be given within 72 hours or immunoglobulin should be given within 6 days when available.

Morbidity and Mortality

Measles case fatality ratios vary, depending upon the average age of infection, nutritional and immunological status of the population, measles vaccine coverage, and access to health care. In developed countries, fewer than 1 in 1,000 children with measles die. In areas of endemicity in sub-Saharan Africa, the measles case fatality proportion may be 5% or higher. Measles is a major cause of child deaths in refugee camps and in internally displaced populations. Measles case fatality proportions in children in humanitarian emergencies, such as refugees, have been as high as 20% to 30% (62). Consequently, a frequent, early response in humanitarian emergencies is to administer measles vaccine.

The measles case fatality ratio is highest at extremes of age. Exposure to an index case within the household may result in more severe disease, perhaps because of transmission of a larger inoculum of virus (63). Vaccinated children, should they develop disease after exposure, have less severe disease and significantly lower mortality rates. Vaccination programs, by increasing the average age of infection, shift the burden of disease out of the age group with the highest case fatality (infancy), further reducing measles mortality.

Measles and malnutrition have important bidirectional interactions. Measles is more severe in malnourished children. Children with severe malnutrition, such as those with marasmus or kwashiorkor, are at particular risk of death following measles. Measles can in turn exacerbate malnutrition by decreasing intake (particularly in children with mouth ulcers), increasing metabolic demands, and enhancing gastrointestinal loss of nutrients as a consequence of a protein-losing enteropathy. Measles in persons with vitamin A deficiency leads to severe keratitis, corneal scarring, and blindness (64).

Measles mortality may be higher in girls than boys (65), although older historical data and more recent surveillance data from the United States do not support this conclusion (66). Supporting the hypothesis of biologic differences in the response to MeV was the observation that girls were more likely than boys to have delayed mortality following receipt

of high-titer measles vaccine (67). The underlying mechanisms are likely differences in immune responses to MeV between girls and boys, although no cogent explanation has been developed.

In regions of high HIV prevalence and crowding, such as urban centers in sub-Saharan Africa, HIV-infected children may play a role in sustaining MeV transmission. Children born to HIV-infected mothers have lower levels of passively acquired maternal antibodies and are thus susceptible to measles at an earlier age than children born to uninfected mothers (53, 68). Protective antibody levels can wane within 2 to 3 years in vaccinated HIV-infected children (69). Children with defective cell-mediated immunity can develop measles without the characteristic rash (70). HIV-infected children have prolonged shedding of MeV RNA (59, 60), potentially increasing the period of infectivity. Counteracting the epidemiologic effects of increased susceptibility of HIV-infected children to measles is their high mortality rate, particularly in sub-Saharan Africa, such that these children do not live long enough for a sizeable pool of susceptible children to develop (71). However, increasing access to antiretroviral therapy increases survival without restoring measles immunity (72). The WHO recently recommended that HIV-infected children receiving antiretroviral therapy receive an additional dose of measles vaccine to protect the individual child and prevent the build-up of susceptible children (73).

PATHOGENESIS IN HUMANS

Incubation Period

The incubation period for measles, the time from infection to clinical disease, is approximately 10 days to the onset of fever and 14 days to the onset of rash. A systematic review estimated the median incubation period from infection to the first onset of signs and symptoms to be 12.5 days (95% confidence interval, 11.8 to 13.2 days) based on 55 observations from eight studies (74). The incubation period may be shorter in infants or following a large inoculum of virus, and it may be longer (up to 3 weeks) in adults.

Virus Replication

Infection is initiated when MeV reaches cells in the respiratory tract, oropharynx, or conjunctivae (Fig. 3). The lower respiratory tract is more susceptible than the nasopharynx, which is more susceptible than the oral mucosa. Direct observations pertaining to the early multiplication of MeV in humans are lacking, but experimental studies in monkeys and experimental and histopathological observations in humans suggest that during the first 2 to 4 days after infection, MeV proliferates locally in the respiratory mucosa and spreads, perhaps within infected pulmonary macrophages and dendritic cells (75), to draining lymph nodes where further replication occurs.

Virus then enters the bloodstream in infected leukocytes, primarily monocytes (76), producing the primary viremia that disseminates infection to sites throughout the reticuloendothelial system. When MeV is administered parenterally, bypassing the usual respiratory route, the incubation period is shortened by 2 to 4 days, suggesting that during natural measles the virus is initially confined to tissues at the portal of entry for this period. Lymphoid tissues throughout the body, including tonsils, adenoids, submucosal lymphoid tissue in the respiratory and gastrointestinal tracts, lymph nodes, thymus, spleen, appendix, and Peyer's patches, become major sites of virus replication. Though clinically inapparent, MeV replication at these sites is indicated by lymphoid hyperplasia and the formation of multinucleated giant cells. In the thymus, MeV infection of epithelial cells leads to apoptosis of uninfected thymocytes and a decrease in the size of the thymic cortex.

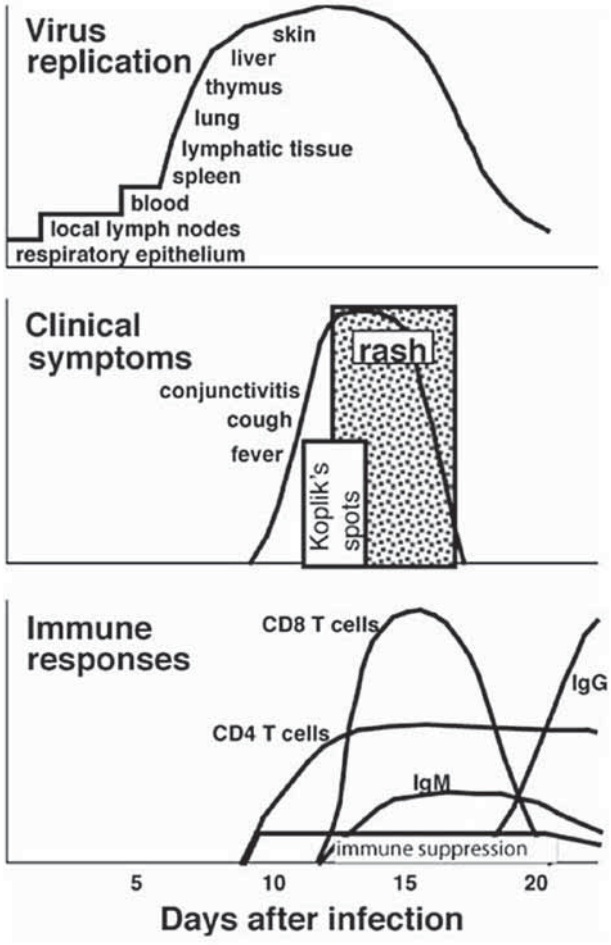

FIGURE 3 Basic pathogenesis of MeV infection. Panels summarize features of the pathogenesis of MeV infection. (a) The spread of the virus from the initial site of infection in the respiratory tract to the skin. Sites of infection are overlaid with virus titer. (b) The appearance of clinical signs and symptoms in relation to viral replication and the immune responses. (c) The immune responses to measles virus. The clinical manifestations arise coincident with the onset of the immune response. (From reference 127 with permission of the publisher.) Source: Moss WJ, Griffin DE. Global measles elimination. *Nat Rev Microbiol* 2006;4:900–908.

Further replication results in a secondary viremia that begins 5 to 7 days after infection and disseminates MeV to tissues throughout the body, including the skin, conjunctivae, CNS, oropharynx, respiratory mucosa, lungs, genital mucosa, kidneys, gastrointestinal tract, and liver (Fig. 3). Replication of MeV in these target organs, together with the host immune response, is responsible for the prodromal signs and symptoms that occur 8 to 12 days after infection and mark the end of the incubation period. The prodromal manifestations reflect involvement of epithelial surfaces in the oropharynx, respiratory tract, gastrointestinal tract, and conjunctivae. During this secondary viremia, virus is again

transported within monocytes and lymphocytes, of which more than 5% may be infected. In a rhesus macaque model, the predominant cell types infected by MeV are $CD150^+$ cells and dendritic cells (77).

Infection of vascular endothelial cells plays a central role in measles pathogenesis. Infection of the endothelial cells of small vessels in the lamina propria and dermis during the secondary viremia precedes infection of the overlying epithelium, and inflammatory changes in and around these vessels are an integral part of the local pathology and characteristic rash.

MeV may also enter respiratory epithelial cells from infected lymphocytes and monocytes through the basolateral surface (78). Virus then buds from the apical surface, allowing for respiratory transmission.

Pathology

The most striking and consistent pathological feature of MeV infection is the formation of multinucleated giant cells that result from the fusion of infected cells with infected and uninfected neighboring cells (Fig. 4). Endothelial cells of small vessels show evidence of MeV infection, including inclusion bodies, MeV antigens, or MeV RNA, during the prodrome and the first days of rash. This is accompanied by vascular dilatation, perivascular infiltration with mononuclear cells, and increased vascular permeability. Changes in the skin, conjunctivae, and mucous membranes of the respiratory and gastrointestinal tracts are secondary to changes in the underlying small blood vessels. MeV antigen and RNA also have been detected in perifollicular histiocytes in lymph nodes and spleen, in epithelial cells of Hassall's corpuscles in the thymus, in biliary duct epithelial cells in the liver, in the epithelium of submucosal glands in the respiratory and gastrointestinal tracts, and in the cells lining hair follicles and sweat glands in the skin.

The rash and Koplik's spots of measles have similar pathogenesis and histopathology (Fig. 4). The initial event is infection of endothelial cells in superficial vessels in the dermis. The earliest histopathologic changes are mild hyperemia, edema, and lymphocytic infiltration of the dermis, with swelling and proliferation of endothelial cells in capillaries, precapillary vessels, and small veins. MeV antigens and viral nucleocapsids can be detected in these endothelial cells on the day before and on the first day of the rash (79). Infection of the overlying epidermis is due to the spread of virus from the infected vascular endothelial cells in the subjacent dermis and by infiltration of infected leukocytes. This leads to the formation of epithelial giant cells containing eosinophilic intracytoplasmic and intranuclear inclusion bodies. A lymphohistocytic infiltrate accumulates around the dilated dermal vessels, and the infected epithelial cells become necrotic. By the third day, this process results in the formation of a vesicle under the stratum corneum that undergoes desiccation and desquamation. In the mucous membranes of the mouth, the necrotic epithelial cells of Koplik's spot slough, leaving a tiny shallow ulcer.

During the secondary viremia, infection of capillary endothelial cells throughout the respiratory tract produces foci of peribroncheolar inflammation, dilated submucosal vessels, perivascular and interstitial mononuclear cell infiltrates, and epithelial hyperplasia. Epithelial giant cells develop in the mucosa from the trachea to the alveoli, and some are shed into the lumen. This pathologic process is well developed by the onset of the prodrome and accounts for the cough and coryza. The damage to the respiratory tract caused by MeV infection also predisposes it to secondary bacterial

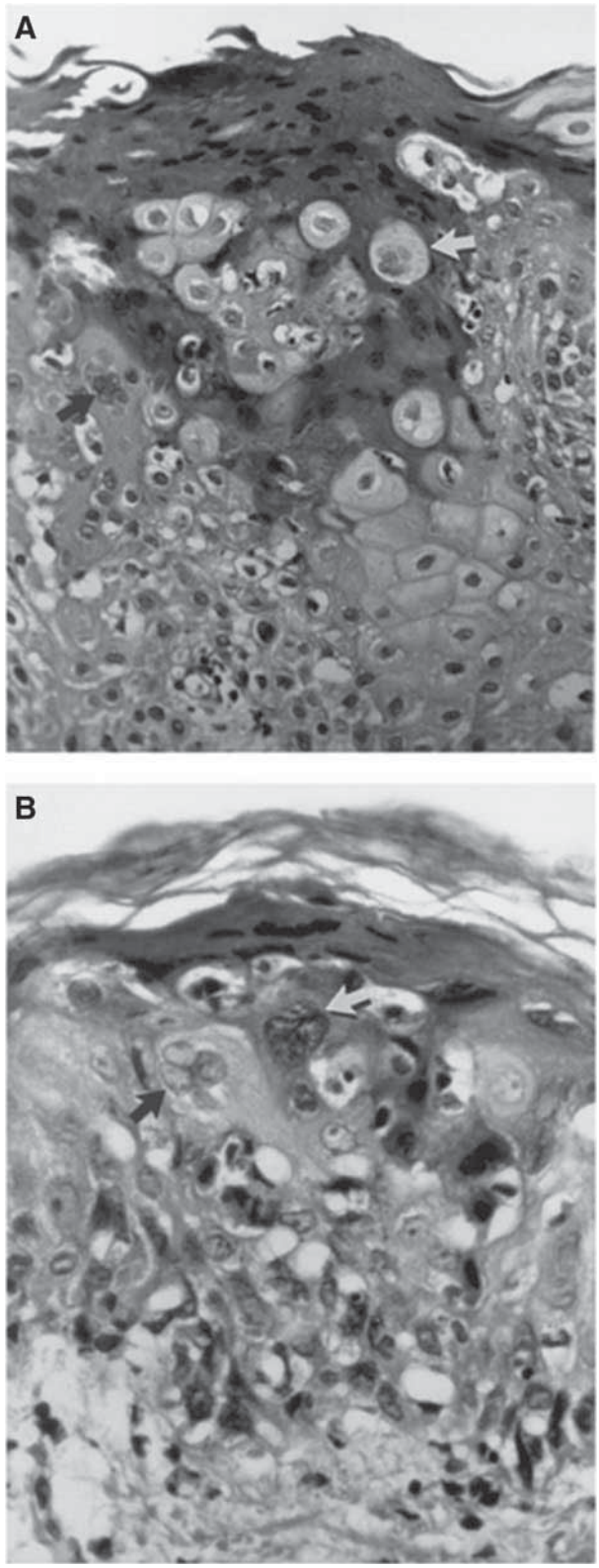

FIGURE 4 Histopathology of Koplik's spots (A) and the skin rash (B) of measles. The epidermal changes in both are characterized by multinucleated giant cells (arrows), focal parakeratosis, dyskeratosis and spongiosis, intracellular edema, and a sparse lymphocytic infiltrate. (Courtesy of D. W. R. Suringa, Tampa, FL). [adapted from Oxman, 2nd edition of *Clinical Virology*.]

infections. In the normal host, virus replication and giant cell formation cease within 2 or 3 days after the onset of the rash, and measles giant cells disappear from the respiratory tract shortly thereafter. With more severe disease, multinucleated giant cells lining the alveoli constitute Hecht's giant cell pneumonia.

Immune Responses

MeV-specific immune responses are essential for recovery from measles and for the establishment of long-term immunity to reinfection, but they also play a role in the pathogenesis of measles and its complications (80, 81). Immune responses to MeV are first apparent during the prodrome and are well developed by the onset of rash. Marked activation of the immune system is manifested by T- and B-cell activation, spontaneous proliferation of peripheral blood mononuclear cells, and increased levels of cytokines and soluble cell surface proteins in the circulation (Fig. 3). Immune suppression, evidenced by impaired delayed type hypersensitivity (DTH) skin test responses to recall antigens and by reduced humoral and cellular immune responses to new antigens, occurs at the same time, and susceptibility to secondary infections is increased. Immune activation and suppression persist for many weeks after recovery from measles.

Innate Immune Responses

In contrast to many other acute virus infections, neither type I nor type III interferon (IFN) is induced as a part of the innate response to MeV infection (82–85). This is likely due to the combined effects of RNA encapsidation and efficient shutdown of IFN induction and response by the C and V nonstructural proteins (18, 86). Suppression of IFN responses may facilitate systemic spread of virus during the clinically silent incubation period.

Humoral Immune Responses

The onset of clinically apparent disease coincides with the appearance of MeV-specific adaptive humoral and cellular immune responses (Fig. 3). Antibodies to MeV are detectable at the time of rash onset (87). The isotype is initially IgM followed by a switch to IgG3 and then, in the memory phase, to IgG1 and IgG4 (87). IgM antibodies generally decline to undetectable levels within 6 to 8 weeks. IgG titers rise rapidly, peak within 3 to 4 weeks, then gradually decline, but generally persist for life. IgG is initially of low avidity, but avidity increases steadily over several months (88). IgG1 is efficiently transported across the placenta, and levels of antibody to MeV are often higher in the newborn than in the mother. The role of mucosal immunity to MeV is unclear. IgA, IgM, and IgG antibodies to MeV are found in secretions, and sampling of saliva has provided a noninvasive method for determining immune status (89, 90).

The most abundant and most rapidly produced antibody is to N. Because of the abundance of anti-N antibody, absence of this antibody is a reliable indicator of seronegativity. The M protein elicits only small amounts of antibody, except in atypical measles. Antibodies to H are the primary antibodies measured by tests based on neutralization of virus infectivity (91). Human convalescent sera show reactivity to linear epitopes as well as to epitopes dependent on conformation and glycosylation. Major conformational and neutralizing epitopes have been localized to an exposed region of the protein without N-linked carbohydrates (7, 92, 93). Antibodies to F contribute to virus neutralization, probably by preventing fusion of the virus membrane with the cell membrane at the time of virus entry (94).

Antibody can protect against MeV infection and may contribute to recovery from infection. Antibody is sufficient for protection because infants are protected by maternal antibody and the level of maternal antibody correlates with failure of the humoral response to vaccination (50). Furthermore, passive transfer of immune serum can partially protect children from measles after exposure. The best correlate of protection is the level of neutralizing antibody, with a plaque reduction neutralizing titer of 120 mIU/ml generally considered the level needed for protection from disease in outbreaks and 1,000 mIU/ml the level needed for protection from infection (95).

IgG antibodies to MeV are maintained for decades (96). MeV extensively replicates in lymphoid tissues during the acute phase of measles, and although infectious MeV is cleared at the time of the rash, MeV RNA persists in blood leukocytes and lymphoid tissues for months. Slow MeV clearance and prolonged immune stimulation by MeV proteins likely contribute to maturation and maintenance of memory immune responses after infection (60, 97).

Contributions of antibody to virus clearance are less clear, but mathematical modeling suggests that they are important (97). Furthermore, failure to mount an adequate antibody response carries a poor prognosis, and levels of antibody-dependent cellular cytotoxicity correlate with clearance of the cell-associated viremia (98). Antibody binding to infected cells alters intracellular virus replication and may contribute to control of infection (99). However, transient depletion of B cells does not affect virus clearance in infected monkeys (100), and children with congenital agammaglobulinemia, who produce no detectable antibody to the virus, recover from infection (101). Such evidence suggests that cell-mediated immunity is essential for MeV clearance.

Cellular Immune Responses

A vigorous T-cell response is induced during MeV infection (97) (Fig. 3). MeV-specific and proliferating $CD8^+$ T cells with evidence of clonal expansion are detectable in blood at the time of the rash. Further, they can be detected in bronchoalveolar lavage fluid during pneumonitis (102). IFN-γ, soluble CD8, and β2 microglobulin are increased in plasma (103, 104), and $CD8^+$ T-cell memory is established after infection (102, 105, 106). Depletion of $CD8^+$ T cells in infected monkeys impairs control of virus replication (107). T-cell epitopes have been identified within all MeV proteins except V, although H contains the majority of epitopes recognized by HLA-A2-positive humans (105, 108).

$CD4^+$ T cells are also activated in response to MeV infection. $CD4^+$ T cells proliferate during the rash, and soluble CD4 is elevated in plasma during acute disease and remains so for several weeks after recovery (109). MeV-specific T-cell proliferation and the production of cytokines are stimulated during measles, and $CD4^+$ T-cell memory is established after recovery.

MeV-specific T cells are responsible for production of a variety of cytokines and soluble factors during disease and recovery. Plasma levels of IFN-γ, neopterin (a product of IFN-γ-activated macrophages), and soluble interleukin (IL)-2 receptor rise during the prodrome, prior to the appearance of the rash (82, 103). This is followed by increases in IL-2 at the time of the rash. As the rash fades, IL-4, IL-10, and IL-13 increase, and elevation of these cytokines persists in some individuals for weeks (104, 110). This pattern of cytokine

production suggests early activation of $CD8^+$ (IFN-γ) and type 1 $CD4^+$ (IFN-γ and IL-2) T cells during the rash followed by activation of type 2 $CD4^+$ T cells (IL-4, IL-13) and then regulatory T cells (IL-10) during recovery. IFN-γ can suppress MeV replication and likely has an important direct antiviral effect.

The cellular immune response is necessary for development of the characteristic measles rash. Biopsies show infiltration of $CD4^+$ and $CD8^+$ T cells and macrophages into areas of virus replication and individuals with deficiencies in cellular immunity may develop severe measles without a rash (111, 112).

MeV-Induced Immunosuppression

The intense immune responses induced by MeV infection are paradoxically associated with depressed responses to unrelated (non-MeV) antigens, lasting for several weeks to months beyond resolution of the acute illness (113). This state of immune suppression enhances susceptibility to secondary bacterial and viral infections that cause pneumonia and diarrhea, and it is responsible for much measles-related morbidity and mortality. DTH responses to recall antigens, such as tuberculin, are suppressed (114), and cellular and humoral responses to new antigens are impaired. Reactivation of tuberculosis and remission of autoimmune diseases after measles have been attributed to this state of immune suppression.

Abnormalities of both the innate and adaptive immune responses have been described following MeV infection (Fig. 5) (115, 116). Transient lymphopenia affecting both $CD4^+$ and $CD8^+$ T lymphocytes occurs in children with measles (117). Functional abnormalities of immune cells are also detected, including decreased lymphocyte proliferative responses (118). The dominant type 2 cytokine response in children recovering from measles can inhibit type 1 responses and increase susceptibility to intracellular pathogens (109). The production of IL-12, important for the generation of type 1 immune responses, decreases following binding of the CD46 receptor (119) and is low for several weeks in children with measles (120), potentially resulting in a limited type 1 immune response to other pathogens. Elevated plasma levels of IL-10, a cytokine capable of inhibiting immune responses, also suggest a role for immunomodulatory cytokines in the immune suppression following measles (68).

CLINICAL MANIFESTATIONS

Typical Measles

The prodromal phase of measles begins with fever, malaise, and anorexia followed by coryza, conjunctivitis, and cough. The catarrhal symptoms increase in intensity, as does the fever, reaching a peak at the height of the skin eruption on about the fifth day. Coryza can be intense, with a profuse mucopurulent nasal discharge. The cough can be severe with a brassy barking quality. Sore throat, eye pain, headache, and myalgia can occur, especially in adolescents and adults. Ocular findings include palpebral conjunctivitis with lacrimation, edema of the lids, photophobia, and punctate keratitis visible on slit lamp examination or with fluorescein staining.

Two to three days before the onset of the rash, Koplik's spots, the pathognomonic enanthem of measles, appear as small (1 mm) white lesions on the buccal mucosa that allow the astute clinician to diagnose measles prior to the onset of rash (Fig. 6). Initially, only a few are present opposite the second molars but these can increase to coat the entire buccal mucosa. Usually by the third day of rash the lesions slough, the erythema fades, and the mucosal membranes regain their normal appearance. Koplik's spots may not be recognized unless the buccal mucosa is examined carefully; they may also be seen on the conjunctivae and other mucosal surfaces, including the gastrointestinal tract.

The intense inflammation of lymphoid tissues during the prodrome can result in generalized lymphadenopathy and mild splenomegaly, with posterior auricular, cervical, and occipital lymph nodes typically enlarged and tender. Lymphoid inflammation is also responsible for the most frequent

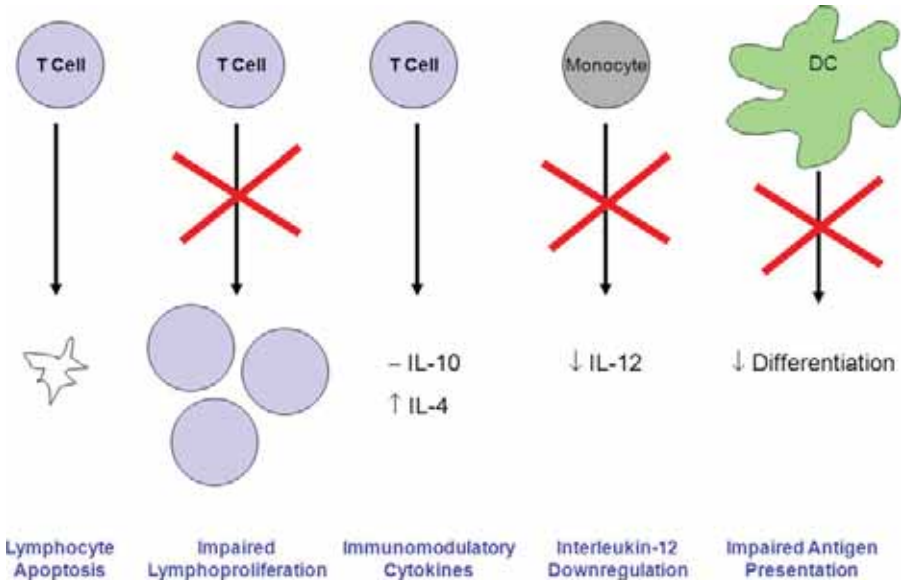

FIGURE 5 Potential mechanisms of immune suppression following measles virus infection. (From reference 119 with permission of the publisher.) Source: Moss WJ, Ota MO, Griffin DE. Measles: Immune suppression and immune responses. *Int J Biochem Cell Biol* 2004;36:1380–1385.

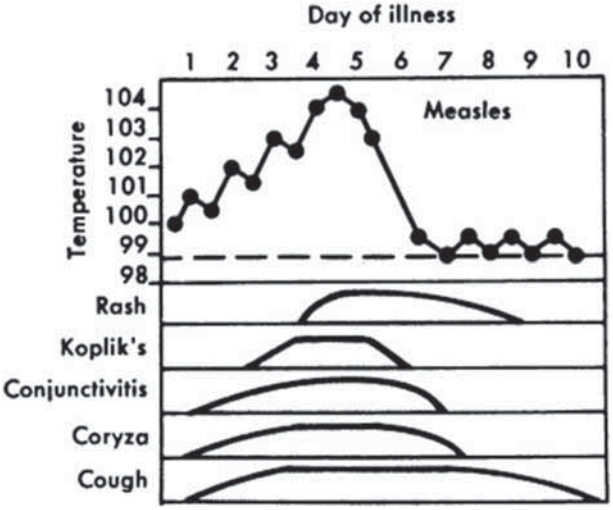

FIGURE 6 Schematic diagram of the clinical course of a typical case of measles. (Adapted from Oxman, 2nd edition of *Clinical Virology*.)

abdominal complication of measles, acute nonsuppurative appendicitis, which can develop prior to the rash.

The rash begins 3 or 4 days after the onset of prodromal symptoms. The earliest lesions, consisting of 3- to 4-mm dull red blanching maculopapules, appear behind the ears, on the forehead at the hairline, and on the upper part of the neck. The rash then spreads downward over the face, neck, upper extremities, and trunk, and it continues downward until it reaches the feet by the third day. The rash is most profuse in the areas first affected. The lesions on the face and neck tend to become confluent, producing a blotchy appearance characteristic of measles (Fig. 7), whereas those on the abdomen and limbs tend to be more discrete. In severe cases, the rash may be associated with edema, especially of the face. The rash begins to fade by the third day in the order of its appearance, so that the rash may be fading on the face by the time it appears on the legs. The fading rash can leave a brownish discoloration of the skin, probably the result of capillary hemorrhage, which resolves during the next 10 days with fine desquamation that usually spares the hands and feet. In fair-skinned children the rash may become purpuric. This type of eruption is not related to severe hemorrhagic measles or the thrombocytopenic purpura that occasionally occurs after the rash has disappeared. Severely undernourished children and children infected with HIV may have a more severe, desquamating rash at the end of the acute illness. In uncomplicated measles, fever reaches a peak of 39° to 40°C at the height of the skin eruption with rapid defervescence on the third or fourth day of rash (Fig. 6).

FIGURE 7 Measles rash. Note the characteristic blotchy appearance. (From reference 127 with permission of the publisher.) Source: Moss WJ, Griffin DE. Global measles elimination. *Nat Rev Microbiol* 2006;4:900–908.

Tracheobronchitis and peribronchial interstitial pneumonitis are common features of uncomplicated measles. Pulmonary infiltrates may be seen on chest radiographs during the acute phase of measles. The illness typically reaches its climax between the second and third days of rash. The temperature then falls rapidly over the following 24 to 48 hours, the coryza and conjunctivitis clear, and the cough decreases in severity, although it may persist for a week or more. Most children in developed countries fully recover within a few days. Persistence or recurrence of fever beyond the third day of rash usually indicates a secondary bacterial infection. Although uncommon in children, hepatic dysfunction has been frequently documented in adults with measles (121). These abnormalities are generally subclinical and self-limited. Myositis, manifested by myalgia and elevated levels of creatine phosphokinase, is observed in 30% to 40% of adolescents and adults during the acute phase of measles, and one-third have hypocalcemia (122).

Modified Measles

Modified measles occurs in partially immunized persons, including persons given immunoglobulin following exposure to MeV and infants with residual maternal antibody. Modified measles is usually a mild version of measles, although the incubation period may be prolonged up to 21 days. The prodrome is shortened or absent, fever is reduced, Koplik's spots are fewer, the rash is short-lived and markedly attenuated, and complications are extremely rare. Patients with modified measles rarely transmit MeV to others.

Atypical Measles

A severe atypical measles syndrome was observed in recipients of formalin-inactivated measles vaccine (FIMV) who were subsequently exposed to wild-type MeV (123). An estimated 600,000 to 900,000 children were immunized with FIMV between 1963 and 1967. After exposure to wild-type MeV, vaccinated individuals were at risk of developing high fever, headache, myalgia, abdominal pain, anorexia, nonproductive cough, and dyspnea followed by the development of an atypical rash that began on the palms and soles and spread centripetally to the proximal extremities and trunk, sparing the face. The rash was initially erythematous and maculopapular but frequently progressed to vesicular, petechial, or purpuric lesions. Most patients had pneumonitis with interstitial infiltrates and segmental pulmonary consolidation, and many had pleural effusions, hilar adenopathy, and nodular parenchymal lesions. Hepatocellular enzymes were often markedly elevated, and some patients had evidence of myositis and disseminated intravascular coagulation. Cases of atypical measles were reported many years after administration of FIMV, but the clinical characteristics in adults were more variable than in children. Despite its severity, atypical measles was self-limited, although pulmonary abnormalities persisted.

In rhesus macaques, the FIMV induces a poor cytotoxic T-cell response and antibody that does not undergo affinity maturation (124, 125). Low-avidity antibody can neutralize infection with viruses that use CD46 as a receptor *in vitro*, as routinely measured by plaque reduction neutralization assays in Vero cells, but cannot neutralize infection with wild-type viruses that primarily use CD150 (125). This difference in neutralization properties may be due to the higher affinity interaction between MeV and CD150 compared to MeV and CD46. Subsequent infection with MeV induces an anamnestic antibody response in both humans and macaques, but the antibody produced is also of low avidity and cannot neutralize wild-type virus. This leads to formation of complexes of nonneutralizing antibody and MeV resulting in immune complex deposition, vasculitis, and pneumonitis in rhesus macaques (124, 125). The exact nature of the defect in immune priming exhibited by FIMV has not yet been identified.

Measles During Pregnancy and the Neonatal Period

Morbidity and mortality are increased in pregnant women with measles, due to an increased risk of MeV pneumonia during the third trimester and peripartum period. Measles during pregnancy has not been associated with congenital abnormalities in the fetus but is associated with an increased incidence of premature delivery and spontaneous abortion (126). Congenital measles, in which the rash is present at birth or appears during the first 10 days of life, varies from a mild illness to a rapidly fatal disease. In the absence of immunoglobulin prophylaxis, the overall mortality is about 30%. Mortality is higher in premature than in term infants and in infants who fail to develop rash (126). Postnatally, replication of wild-type MeV in permissive cells results in characteristic cytopathic effects that include the formation of multinucleated giant cells as well as the production of eosinophilic intranuclear and intracytoplasmic inclusion bodies. Eosinophilic Cowdry type A intranuclear inclusion bodies are characteristic of morbillivirus infection. Acquired measles in the neonate is rare because passively acquired maternal antibodies result in protection of most newborns. In the absence of protective maternal antibodies, however, measles in neonates is often severe.

Measles in Immunocompromised Patients

Children and adults with deficient cell-mediated immunity may develop severe, progressive, and frequently fatal MeV infection, often in the absence of the typical rash and characteristic prodrome. The most frequent manifestation is giant cell pneumonia (Hecht pneumonia), characterized by increasing respiratory insufficiency, progressive interstitial pneumonia with multinucleated giant cells throughout the tracheobronchial and alveolar epithelium (Fig. 8), the presence of measles giant cells in pulmonary and nasopharyngeal secretions, and a chest radiograph showing diffuse interstitial and alveolar infiltrates resembling adult respiratory distress syndrome (70, 127). The case fatality ratio for MeV pneumonia was estimated to be about 70% in oncology patients and about 40% in HIV-infected patients in one early study (127). However, mortality in HIV-infected children was more recently estimated to be 10% to 15% (128).

The other frequent manifestation of progressive MeV infection in immunocompromised patients is measles inclusion body encephalitis (MIBE). MIBE may accompany or follow giant cell pneumonia, but it more often occurs as the sole clinical manifestation months after MeV infection (129). The disease usually presents with refractory focal myoclonic seizures and altered mental status and progresses to generalized seizures, coma, and death. Mortality exceeds 85%. Patients with MIBE are frequently afebrile and have normal cerebrospinal fluid (CSF) analysis and head computed tomography and magnetic resonance imaging scans. Electroencephalograms are abnormal but nonspecific. Progression is often rapid, with the majority of deaths occurring within 6 to 8 weeks of onset. Survivors have severe neurological sequelae. At autopsy or biopsy, the brain shows gliosis and focal necrosis, lymphocytic perivascular cuffing, and eosinophilic intranuclear and intracytoplasmic inclusions in glial cells and neurons. MeV antigens are detectable by immunofluorescent staining, and MeV RNA can be detected by reverse transcriptase-PCR (RT-PCR). Live attenuated measles vaccine may also cause MIBE in severely immunodeficient infants (130).

Measles also is severe in malnourished children, frequently resulting in secondary infections causing pneumonia and diarrhea, and with a case fatality ratio exceeding 10%. Many factors are likely to contribute to the increased morbidity and mortality, including early age of infection, rapid loss of maternal antibody, vitamin A deficiency, and prior

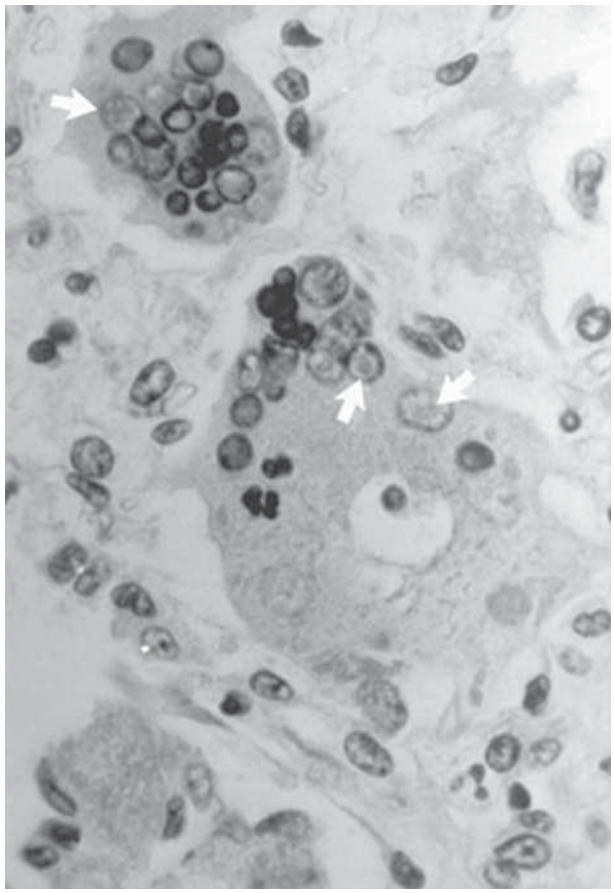

FIGURE 8 Measles giant cell pneumonia. Two multinucleated epithelial giant cells are visible in alveolar spaces in the lung of an immunosuppressed child who died of giant cell pneumonia. Eosinophilic Cowdry type A inclusion bodies are visible in many nuclei (arrows). (From reference 56 with permission of the publisher.) [Adapted from Oxman, 2nd edition of *Clinical Virology*.]

or concurrent infection with other pathogens. However, depressed cell-mediated immune responses secondary to malnutrition likely contribute significantly to high risk of morbidity and mortality.

Complications

In developed countries, about 10% of measles cases are associated with complications, although the rate exceeded 20% during the 1989 to 1990 measles epidemic in the United States, primarily because of the high proportion of cases in young children and adults. The most common complications are otitis media (5% to 9%), diarrhea (5% to 9%), pneumonia (1% to 7%), and encephalitis (0.1%). Pneumonia is more common in young children, and encephalitis more common in adolescents and adults. In resource-poor countries, measles is a devastating disease with complication rates as high as 80% in many epidemics, and case fatality rates may exceed 10%. Diarrhea and pneumonia are frequently fatal. Keratitis in children with vitamin A deficiency can lead to corneal ulceration and blindness, and secondary bacterial infections cause otitis media, osteomyelitis, and other pyogenic complications.

Respiratory Tract Complications

The most frequent complications of measles involve the respiratory tract. Coryza, mild laryngitis, and tracheobronchitis with cough are almost invariably present in uncomplicated measles. Only when they are unusually severe or prolonged are they considered complications. Pneumonia is the most frequent life-threatening complication of measles (131), and it may present clinically as bronchiolitis in infants or as bronchopneumonia or lobar pneumonia in infants and older children. Although the incidence in developed countries is less than 10%, pneumonia accounts for more than 60% of measles-associated deaths. In children, pneumonia is usually caused by secondary bacterial infection and occurs predominately in children younger than 5 years of age. Common bacterial pathogens are *Streptococcus pneumoniae* and *Haemophilus influenzae* type b (Hib) in unvaccinated children and *Staphylococcus aureus*. Widespread use of Hib and pneumococcal conjugate vaccines should decrease the incidence of these pathogens as causes of postmeasles pneumonia. Secondary bacterial pneumonia should be suspected in any child with measles who develops respiratory distress in association with persistence or recurrence of fever.

Symptomatic pneumonia is observed in 5% to 15% of immunocompetent adults with measles. In contrast to pneumonia in children, pneumonia in adults is more commonly caused by MeV itself. Respiratory distress and hypoxemia develop in parallel with the rash, and there is evidence of prolonged and extensive MeV replication, including high and persistent fever, persistence of rash, persistent viremia, and laboratory evidence of hepatitis and myositis (121). Pneumonia caused by bacterial superinfection develops later, usually 5 to 10 days after the onset of the rash. Typically, deteriorating clinical and pulmonary status, high fever, elevated white blood cell count, and purulent sputum develop in a patient whose rash is resolving. In MeV pneumonia, chest radiographs reveal bilateral diffuse reticulonodular interstitial infiltrates. Segmental pulmonary consolidation, parenchymal nodules, hilar adenopathy, and pleural effusions are rarely observed except in atypical measles. Measles pneumonia is frequently a severe illness in healthy young adults, but fatalities are rare.

Otitis media is usually signaled by increasing irritability and ear pulling in infants or earache in older children, together with persistence or recurrence of fever. In severe cases, the first sign of otitis media may be spontaneous perforation of the tympanic membrane with a purulent discharge from the middle ear. The incidence of otitis media is increased in infants.

Laryngotracheobronchitis (croup) due to MeV may occur in up to 20% of children with measles who are under 2 years of age, and it should be suspected when a child with measles develops inspiratory stridor, progressive hoarseness, a barking cough, and suprasternal retraction. Increased restlessness, dyspnea, anxiety, and tachycardia suggest increasing airway obstruction (obstructive laryngitis), which may require tracheostomy. Bronchiolitis may also complicate measles in infants and children younger than 2 years of age and is clinically indistinguishable from bronchiolitis caused by respiratory syncytial virus. Sinusitis occurs in 2% to 4% of patients with measles, primarily in adolescents and adults, and has been observed in 25% of young adults hospitalized with pneumonia.

Neurological Complications (Table 2)

Uncomplicated measles is frequently accompanied by CSF pleocytosis and electroencephalographic abnormalities, but there is no evidence that the parenchyma of the brain is directly infected. Infection of vascular endothelial cells is a central feature of the pathogenesis of uncomplicated measles, and vascular endothelial cells in the brain are not spared (132). MeV infection of vascular endothelial cells in the CNS provides a route of entry for virus into the brain parenchyma in those rare patients who develop MIBE or subacute sclerosing panencephalitis (SSPE).

Acute Postinfectious Measles Encephalomyelitis

Acute postinfectious measles encephalomyelitis is the most common neurological complication of measles. It is rare in children under 2 years of age, but it occurs in about one in 1,000 cases of measles in older children and somewhat more frequently in adults (133). The onset is usually during the first week after appearance of the rash, but it

TABLE 2 Neurologic complications of measles

Disease	Host	Typical age of measles	MeV in brain	Incidence	Pathology	Time course
Acute postinfectious measles encephalitis	Normal	>2 years	No	1:1,000 cases	Demyelination and perivascular inflammation	Monophasic course over weeks
Measles inclusion body encephalitis (MIBE)	Immune suppressed	Any	Yes	Unknown	Inclusion bodies in neurons and glial cells	Progressive course over months
Subacute sclerosing panencephalitis (SSPE)	Normal	<2 years	Yes	1:10,000 cases	Inclusion bodies and inflammation	Progressive course over years

Adapted from reference 26.

occasionally develops during the prodrome. Onset is typically abrupt, starting with resurgent fever, irritability, headache, vomiting, and confusion and progresses rapidly to obtundation and coma. These manifestations are frequently accompanied by seizures, and some patients develop focal neurological signs, including cerebellar ataxia, myelitis, optic neuritis, and retinopathy. There are usually signs of meningeal irritation, and the CSF shows a mild lymphocytic pleocytosis and a moderately elevated protein concentration. Mortality is 10% to 20%, and the majority of survivors have neurological sequelae.

Acute postinfectious measles encephalomyelitis appears to be an autoimmune disease. The neuropathology is similar to that of experimental autoimmune encephalomyelitis, with lymphocytic perivascular cuffing and perivenular demyelination (134). Neither MeV RNA nor viral antigens have been detected in the brain, and there is no intrathecal synthesis of MeV-specific antibody. However, antibodies to myelin basic protein are present in the CSF. The pathogenesis is unclear but possibilities include altered presentation of myelin antigens, activation and expansion of autoreactive lymphocytes as a result of the immune activation and dysregulation, and molecular mimicry. There are regions of homology between MeV proteins and myelin basic protein, but neither cross-reactive antibodies nor cross-reactive T cells have been identified (134).

Subacute Sclerosing Panencephalitis

A second form of measles encephalitis, SSPE, is a rare delayed complication of measles that occurs in approximately one in 10,000 cases (135). Typically, SSPE presents 6 to 8 years after measles that occurred in early childhood, generally before the age of 2 years (136). The onset is insidious, with symptoms of progressive loss of cortical function developing over months. In the early stages, subtle personality changes and declining school performance often indicate deteriorating intellectual capacity. There may also be awkwardness and stumbling followed by development of myoclonic jerks, seizures, and characteristic EEG changes consisting of periodic high-amplitude slow-wave complexes. Patients subsequently develop ataxia, progressive mental deterioration, and extrapyramidal dyskinesias, including choreoathetosis and dystonic posturing. Progressive loss of vision is caused by chorioretinitis, optic atrophy, or cortical blindness. Disease progression is variable and periods of remission are common, but in most cases death occurs within 1 to 3 years of onset (137).

Pathologic examination reveals diffuse encephalitis involving both white and gray matter with perivascular cuffing, diffuse lymphocytic infiltration, extensive microglial proliferation, and patchy demyelination. Neurons and glial cells contain typical nuclear and cytoplasmic inclusion bodies composed of MeV ribonucleocapsids, but multinucleated giant cells are not present. Although large amounts of viral RNA and N and P proteins are present, the MeV envelope proteins are usually markedly reduced, absent, or functionally defective, resulting in deficient virion assembly and budding (138). The defective MeVs in SSPE brains have mutations throughout the genome, especially within the M, H, and F genes, and infectious MeV cannot be recovered (139–141).

Patients with SSPE have exceptionally high titers of antibody to MeV in their plasma and CSF. Synthesis of antibody to MeV by plasma cells in the CNS results in elevated levels of MeV-specific CSF immunoglobulin. Because this antibody is of limited heterogeneity, electrophoretic analysis of the CSF typically reveals oligoclonal bands of IgG (142).

The pathogenesis of SSPE is unclear, but sequence analysis of MeV RNA from various parts of the brain suggests that virus in the CNS is clonal. Neurons and glial cells are not fully permissive for MeV, down-regulate transcription, and increase the 3′-5′ transcription gradient, resulting in a marked reduction in the synthesis of envelope glycoproteins. Infected cells produce MeV nucleocapsids and defective interfering particles, but little or no infectious virus. Antibodies to MeV may contribute to this process by further inhibiting transcription of MeV mRNA and removing MeV proteins from the cell membrane. The result is the establishment of a persistent infection that can slowly progress by cell-to-cell spread within the brain in the absence of giant cell formation. Immune lysis of cells expressing even small amounts of MeV envelope proteins may limit progression of the infection and could account for the prolonged latent period of SSPE. The diagnosis of SSPE is based on (i) the gradual and progressive onset of behavioral changes, myoclonus, dementia, visual disturbances, and pyramidal and extrapyramidal signs; (ii) characteristic periodic EEG discharges; and (iii) demonstration of antibodies to MeV in the CSF.

Measles Inclusion Body Encephalitis

The third form of measles encephalitis, MIBE, is a progressive, generally fatal infection of the brain that occurs in immunocompromised patients (see Measles in Immunocompromised Patients).

Gastrointestinal Complications

MeV infection of epithelial surfaces and lymphoid tissues throughout the gastrointestinal tract is responsible for the nausea, vomiting, diarrhea, and diffuse abdominal pain observed in 30% to 60% of children and adults with measles, as well as for rare cases of nonsuppurative appendicitis. Laboratory evidence of hepatitis occurs in the majority of adults with measles, but there is rarely symptomatic hepatitis or jaundice (121). In developed countries, these manifestations are self-limited and generally resolve as the rash disappears. In developing countries, severe and persistent diarrhea occurs in 20% to 70% of children with measles and is frequently associated with secondary bacterial or protozoal infection (143).

Cardiovascular Complications

Electrocardiographic evidence of myocarditis and pericarditis, including prolongation of the P-R interval, ST segment abnormalities, and T wave inversions, can be detected in 15% to 30% of children and adults during the acute phase of measles (144). These abnormalities are transient and rarely result in symptomatic disease. Prolongation of the Q-T interval also may be observed, presumably related to the transient hypocalcemia that can occur during the acute phase of measles.

Ocular Complications

Conjunctivitis and punctate keratitis are features of uncomplicated measles and resolve as the rash disappears. In malnourished children with vitamin A deficiency, these lesions frequently progress to corneal ulceration, which may be complicated by secondary bacterial infection. Consequently, before the widespread use of measles vaccine measles was an important cause of childhood blindness in developing countries where vitamin A deficiency was prevalent.

Other Complications of Measles

Mild to moderate thrombocytopenia is common during the acute phase of uncomplicated measles, but it is asymptomatic and transient, resolving soon after the rash disappears. Rarely, thrombocytopenic purpura develops several weeks after uncomplicated measles. This complication resembles idiopathic thrombocytopenic purpura and has an excellent prognosis.

Severe hemorrhagic measles (black measles) is an extremely rare form characterized by the sudden onset of high fever, seizures, delirium, respiratory distress, and a confluent hemorrhagic eruption in the skin and mucous membranes. Bleeding from the nose, mouth, gastrointestinal tract, and genitourinary tract are frequently severe and uncontrollable, and mortality is high. The pathogenesis appears to involve disseminated intravascular coagulopathy associated with extensive MeV infection of vascular endothelium.

Tuberculosis may be exacerbated or reactivated by measles, presumably because of the prolonged suppression of cell-mediated immunity induced by MeV infection (114). This has not been observed after live attenuated MeV vaccine, although suppression of tuberculin skin test reactivity and *in vitro* lymphoproliferative responses occur after measles vaccination.

There are conflicting and inconclusive data suggesting that persistent MeV infection causes or contributes to the development of chronic diseases of unknown etiology, including multiple sclerosis, Paget's disease, inflammatory bowel disease, and otosclerosis (66). However, no causal association has been established between MeV or measles vaccines and these conditions.

Clinical Diagnosis

Measles is readily diagnosed on clinical grounds. Koplik's spots are especially helpful because they appear early and are pathognomonic of measles. Clinical diagnosis is more difficult during the prodrome, when the illness and rash are attenuated by passively acquired MeV antibodies or prior immunization, or when the rash is absent in immunocompromised patients and severely undernourished children. Clinical diagnosis is also more difficult in regions where the incidence of measles is low because other pathogens are responsible for the majority of measles-like illnesses (fever and rash). The Centers for Disease Control and Prevention (CDC) case definition for measles requires (i) a generalized maculopapular rash of at least 3 days' duration; (ii) fever of at least 38.3°C (101°F); and (iii) either cough, coryza, or conjunctivitis. While the CDC case definition has a sensitivity of at least 90%, its specificity is only about 25% in the absence of endemic and epidemic measles.

The differential diagnosis of measles includes a number of conditions associated with fever and rash, including rubella, enterovirus infections, drug eruptions and other allergic rashes, scarlet fever, meningococcemia, roseola infantum caused by human herpesvirus 6, erythema infectiosum caused by parvovirus B19, and dengue virus infection. Other diseases that may cause maculopapular rashes resembling measles include toxic shock syndrome, infectious mononucleosis caused by Epstein-Barr virus, toxoplasmosis, and Kawasaki's disease.

Laboratory Diagnosis

Virus Isolation

Measles can be diagnosed by isolating virus in cell culture from respiratory secretions, nasopharyngeal and conjunctival swabs, peripheral blood mononuclear cells, or urine. Spin amplification (shell vial) assays, with fluorescent antibody staining for MeV antigens, can improve the speed and sensitivity of culture diagnosis. Nevertheless, virus isolation remains technically difficult and unavailable in most clinical settings.

Cytologic Diagnosis and Antigen Detection

Direct detection of giant cells in respiratory secretions or urine; on accessible epithelial surfaces, such as the pharynx, nasal mucosa, buccal mucosa, or conjunctiva; or in tissue obtained by biopsy provides a rapid and practical means of diagnosis. Characteristic multinucleated giant cells containing eosinophilic intranuclear and intracytoplasmic inclusion bodies are ordinarily present during the prodrome and for the first 2 or 3 days of the rash. Multinucleated giant cells with eosinophilic intranuclear inclusion bodies are also produced by herpes simplex and varicella-zoster virus infections. Detection of MeV antigens by immunofluorescent or immunoenzyme staining increases sensitivity and specificity. These techniques can detect MeV antigens later in the disease when infectious virus can no longer be isolated. Polyclonal sera and monoclonal antibodies are both effective, but antibodies to the MeV N protein are most useful because this viral protein is the most abundant antigen.

Nucleic Acid Detection

Detection of MeV RNA by RT-PCR amplification of RNA extracted from clinical specimens or tissue can be accomplished using primers targeted to highly conserved regions of the MeV N, M, or F genes. Testing is frequently performed at WHO Regional or Global Network Laboratories, including the CDC. These techniques provide an extremely sensitive and specific means of diagnosis and are especially useful in CNS infections in which infectious virus cannot be readily isolated and in immunocompromised patients who may not be capable of antibody responses. RT-PCR assays can be applied to specimens obtained under field conditions and, when combined with nucleotide sequencing, permit the precise identification and characterization of MeV genotypes for molecular epidemiologic studies.

Serologic Diagnosis

Serology has been the mainstay of the laboratory diagnosis of measles. A 4-fold or greater increase in MeV-specific IgG antibody levels between acute and convalescent sera or the detection of MeV-specific IgM or low-avidity IgG antibodies in a single specimen of serum or saliva are considered diagnostic of acute MeV infection. The presence of IgG antibody to MeV in a single serum specimen is evidence of prior infection or immunization. In primary infection in the normal host, detectable antibodies to MeV generally appear in the serum within 1 to 3 days of rash onset and reach peak levels in 2 to 4 weeks. Because some patients will already have had a substantial rise in antibody titer if the initial serum is obtained 4 days or more after rash onset, acute serum should be obtained as soon as possible after the onset of symptoms. Convalescent serum should be obtained 2 to 4 weeks later, although an interval of 7 days is often sufficient to demonstrate a rising antibody level. MeV-specific IgM antibodies may not be detectable with some currently available assays until 4 to 5 days or more after rash onset, and MeV-specific IgM antibodies usually fall to undetectable levels within 4 to 8 weeks of rash onset (145).

A number of methods are available for measuring antibodies to MeV. Neutralization tests are sensitive and specific, the results are highly correlated with immunity to infection,

and they provide the most clinically relevant measure of response to immunization (146). However, they require propagation of MeV in cell culture and are thus expensive, laborious, and not widely available. The hemagglutination inhibition (HI) test results correlate well with those of conventional neutralization tests and the presence of HI antibody is indicative of immunity to measles. The complement fixation test is less sensitive than HI or neutralization tests and more difficult to perform, and it is now rarely used.

Enzyme immunoassays (EIAs) are now the most widely used means of serologic diagnosis. Employing antigens prepared from MeV-infected cells or recombinant MeV proteins, they can detect both IgM and IgG antibodies. With sensitive EIAs, it is possible to detect MeV-specific IgM antibodies in patients with secondary immune responses to MeV, albeit at lower IgM to IgG ratios than observed in patients with primary immune responses. Thus the presence of IgM antibody is not necessarily indicative of primary MeV infection. Recent MeV infection also can be distinguished from past infection by the presence of low-avidity antibody and IgG3 (147).

PREVENTION

General

Environmental disinfection has little impact on the spread of MeV because the virus is efficiently transmitted by respiratory routes and fomites do not play a significant role in transmission. Quarantine is generally futile because exposure often occurs during the prodrome and before the diagnosis is made. Airborne precautions are indicated for all hospitalized patients with measles until 4 days after the onset of the rash. These include use of an N95 respirator or a respirator with similar effectiveness in preventing airborne transmission and preferably placement in a single patient, airborne infection isolation room (see CDC recommendations for details: http://www.cdc.gov/hicpac/). Immunocompromised patients with measles may continue to shed virus and should be isolated for the duration of their illness. Susceptible medical personnel exposed to measles should be relieved from patient contact from the 5th through the 21st day after exposure, regardless of whether or not they receive postexposure immunization with vaccine or Ig. Personnel who become ill should be relieved from patient contact until 5 days after the onset of rash.

Passive Immunoprophylaxis

Ig can prevent or modify measles in susceptible persons, but administration of measles vaccine is the preferred intervention. Attenuated measles vaccines may provide some protection to immunocompetent persons if administered within 72 hours of exposure and have the advantage of inducing long-term immunity. In immunocompetent persons, administration of Ig within 72 hours of exposure usually prevents MeV infection and almost always prevents clinical measles. Administered up to 6 days after exposure, Ig will still prevent or modify the disease. Prophylaxis with Ig is recommended for susceptible household and nosocomial contacts that are at risk of developing severe measles, particularly children younger than 1 year of age, immunocompromised persons (including HIV-infected persons previously immunized with attenuated measles vaccine), and pregnant women. Except for premature infants, children younger than 6 months of age will usually be partially or completely protected by passively acquired maternal antibody. If measles is diagnosed in a mother, all unimmunized children in the household should receive Ig. The recommended dose of Ig is 0.25 ml/kg of body weight given intramuscularly: immunocompromised persons should receive 0.5 ml/kg. The maximum total dose is 15 ml. Intravenous immunoglobulin (IgIV) contains antibodies to MeV and the usual dose of 100 to 400 mg/kg should provide adequate prophylaxis for measles exposures occurring as long as 3 weeks or more after IgIV administration.

In countries where the use of measles vaccine has been widespread for decades, most adults are immune as a consequence of vaccination rather than natural infection, and the reduction in indigenous MeV transmission has eliminated the immunologic "boosting" associated with reexposure. Consequently, levels of antibody to MeV in adults are considerably lower than they were when wild-type MeV infection was prevalent, resulting in lower levels of passively acquired maternal antibody to MeV in newborns (148) and lower levels of antibody to MeV in current lots of immunoglobulin (149).

Susceptible persons who receive postexposure prophylaxis with Ig should be immunized with attenuated measles vaccine (if it is not contraindicated). Measles vaccine should be given 5 months after Ig if the dose was 0.25 mg/kg (standard dose) and 6 months after Ig if the dose was 0.5 mg/kg (for immunocompromised persons).

Active Immunization

Remarkable progress in reducing measles incidence and mortality has been and continues to be made as a consequence of increasing routine measles vaccine coverage, provision of a second dose of measles vaccine through routine immunization services or supplementary immunization activities (SIAs), and increasing efforts by the WHO, the United Nations Children's Fund (UNICEF), and their partners in the Measles and Rubella Initiative to achieve and sustain measles elimination. All six WHO regions have adopted measles elimination goals, with the latest date being 2020.

This achievement in reducing measles impact attests to the enormous public health significance of measles vaccination and was a key factor in achieving the Millennium Development Goal 4 to reduce overall child mortality. The most recent global measles goals were to achieve the following by 2015: (i) increase coverage with the first dose of measles vaccine to >90% nationally and >80% in every district; (ii) reduce global measles incidence to fewer than five cases per million population; and (iii) reduce global measles deaths by 95% compared to the estimated number of measles deaths in 2000 (40).

Measles Vaccines

The process of adaptation of MeV grown in nonsusceptible host cells, such as the chick embryo and canine and bovine kidney cells, led successfully to the development of attenuated vaccine strains. The first attenuated measles vaccine was developed by passage of the Edmonston strain of MeV in chick embryo fibroblasts to produce the Edmonston B virus (Fig. 9) (150). Licensed in 1963, this vaccine was protective, but also reactogenic, inducing fever and rash in a large proportion of immunized children. Reactions were reduced when Ig that contained antibodies to MeV was given at the time of vaccination.

More extensive passage of the Edmonston B virus in chick embryo fibroblasts produced the more attenuated Schwarz vaccine that was licensed in 1965 and currently

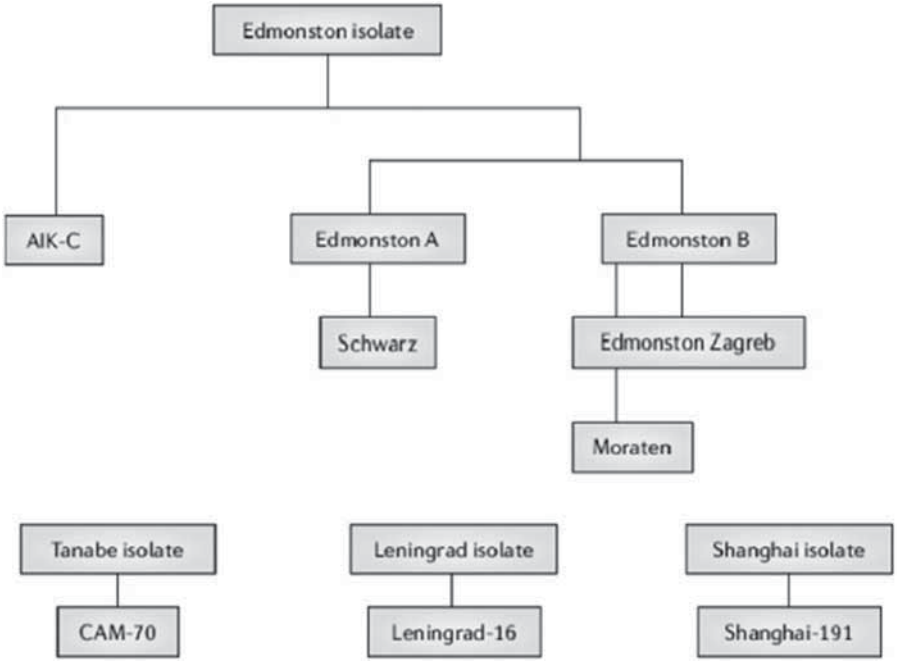

FIGURE 9 Measles virus vaccines. Most attenuated measles vaccines were developed from the Edmonston strain of measles virus. The Edmonston B vaccine was the first licensed measles vaccine but was associated with a high frequency of fever and rash. The further attenuated Schwarz and Edmonston-Zagreb vaccines are widely used throughout the world. The Moraten vaccine is the only measles vaccine used in the United States. [Origin: Markowitz LE. Measles control in the 1990s: immunization before 9 months of age. Document WHO/EPI/GEN/90.3. Geneva: World Health Organization, 1990. Reprinted in: Cutts FT. Measles. Module 7. The Immunological Basis for Immunization Series. Document WHO/EPI/GEN/93.17. Geneva: World Health Organization, 1993.] (From reference 127 with permission of the publisher.) Source: Moss WJ, Griffin DE. Global measles elimination. *Nat Rev Microbiol* 2006;4:900–908.

serves as the standard measles vaccine in much of the world (Fig. 9). The Moraten strain (licensed in 1968 and meaning "more attenuated Enders" strain) used in the United States is closely related to the Schwarz strain (Fig. 9) (151). Other Edmonston-derived vaccine strains (e.g., Zagreb, AIK-C) and attenuated strains developed independently (e.g., CAM, Leningrad-16, Shanghai-191) are also successful vaccines. Few antigenic differences have been described among MeV vaccine strains (all genotype A) regardless of the geographic origin of the parent virus. However, the Edmonston-Zagreb vaccine is produced in human diploid cells, rather than chick embryo fibroblasts, and may be more reactogenic and immunogenic in young infants and when delivered by the aerosol route (152).

The lyophilized attenuated vaccine virus is relatively stable, but the reconstituted vaccine rapidly loses infectivity. The attenuated virus is inactivated by light and heat, and after reconstitution loses about half of its potency at 20°C and almost all potency at 37°C within an hour. Therefore, a cold chain must be maintained prior to and after reconstitution. Attenuated vaccine viruses replicate less efficiently than wild-type MeV but induce both neutralizing antibody and cellular immune responses qualitatively similar to that induced by natural disease, although antibody titers are lower (153). Antibodies first appear 12 to 15 days after vaccination and peak at 1 to 3 months. In many countries, attenuated measles vaccine virus is combined with other live attenuated virus vaccines such as those for rubella (MR), mumps and rubella (MMR), and mumps, rubella, and varicella (MMRV). With increasing global emphasis on rubella elimination, more countries are using combined measles and rubella vaccines.

Administration

The recommended age of vaccination with the first dose of measles-containing vaccine (MCV) varies from 6 to 15 months depending on region and is a balance between the optimum age for seroconversion and the probability of acquiring measles before that age. In areas where measles remains prevalent, measles vaccination is routinely performed at 9 months, whereas in areas with little measles, vaccination is often at 12 to 15 months. During epidemics and among HIV-infected infants, the measles vaccine may be administered at 6 months with another routine dose at 9 months of age.

Attenuated measles vaccine is administered subcutaneously or intramuscularly. However, there is substantial interest in alternate routes of delivery that would not require needles and syringes. Neither oral nor intranasal administration is effective, but delivery to the lower respiratory tract may be more promising. Aerosol administration was advocated by Albert Sabin in the early 1980s, is highly effective in boosting preexisting antibody titers, and may hold promise for use in older children. However, the primary immune response to aerosolized measles vaccine is lower than it is to subcutaneous administration of the same vaccine (154). The reasons for this are not known but may be related to dose or efficiency of delivery and infection. A promising approach is the use of microneedle patches to deliver lyophilized vaccine virus subcutaneously (155).

The proportions of immunized children who develop protective levels of antibody are approximately 85% at 9 months of age and 95% at 12 months of age (156), although these proportions vary by vaccine strain and host characteristics (157). Genetic background affects the likelihood of

seroconversion and antibody titers (158, 159). Common childhood illnesses at the time of vaccination may also reduce immune responses, although this is not frequent and should not be a reason for withholding vaccination (160). Any potential decrease in seroconversion must be balanced against the loss of the opportunity for vaccination and the consequent risk of the child acquiring measles. Similar compromises must be considered with respect to immunizing individuals infected with HIV. Overall, measles vaccine has been well tolerated and immunogenic in HIV-infected children and adults, although antibody levels may wane (69). Because of the potential severity of wild-type MeV infection in HIV-infected individuals (70), attenuated measles vaccine is recommended for routine administration to HIV-infected children, except those who are severely immunocompromised (73, 157). Attenuated measles vaccine is also contraindicated in individuals with other severe deficiencies of cellular immunity because of the possibility of disease due to progressive pulmonary or CNS infection.

The dose of MeV routinely used for immunization is between 10^3 and 10^4 plaque-forming units. When 10- to 100-fold higher doses were used, seroconversion in younger infants improved, and in 1990 the WHO Expanded Program for Immunization (EPI) recommended use of the high-titer Edmonston-Zagreb vaccine at 6 months of age in countries where measles before the age of 9 months was a significant cause of death. However, subsequent follow-up of children receiving high-titer vaccines in countries with high childhood mortality showed an increased mortality in girls over the subsequent 2 to 3 years, and this recommendation was withdrawn (67). Mortality was not due to measles, but rather to a relative increase in the deaths due to other infections. The pathogenesis of delayed increased mortality after the high titer vaccine is not understood but occurred primarily in those who developed a rash after vaccination and may be related to long-term suppression of immune responses similar to that induced by measles or alteration of immune responses associated with a change in the sequence of delivery of vaccines (161).

The duration of vaccine-induced immunity is variable. Secondary vaccine failure rates have been estimated to be approximately 5% at 10 to 15 years after immunization, but they are probably lower when vaccination is given after 12 months of age (162). Waning antibody levels occur within 2 to 3 years of measles vaccination of HIV-infected children in the absence of antiretroviral therapy (69). However, decreasing antibody titers do not necessarily imply a complete loss of protective immunity because a secondary immune response usually develops after re-exposure to MeV, with a rapid rise in antibody titers without overt clinical disease (163).

Second Dose of Measles Vaccine

With increasing emphasis on regional measles elimination and the potential for global measles eradication (see Regional Elimination and Global Eradication), achieving high coverage with two doses of measles-containing vaccine (MCV2) is receiving increased attention. The herd immunity threshold (H) is the level of population immunity necessary to interrupt MeV transmission and is estimated by the equation, $H = 1 - 1/R_0$. This equation is based on several simplifying assumptions, but it provides an approximation of the level of population immunity required to eliminate measles. For MeV, this value is 93% to 95%, a level of population immunity that cannot be achieved with a single dose of MCV at 9 months of age. Thus, a second dose is required for measles elimination. This second dose can be administered through routine immunization services, often in the second year of life or prior to school at 4 to 6 years of age, or through mass vaccination campaigns called supplemental immunization activities (SIAs). In the United States, many colleges and universities require a second dose of measles vaccine because of the shift to older age groups in recent measles outbreaks. This second dose serves to immunize those children who failed to respond to the first dose or who missed it. The vast majority of children who do not develop protective antibodies after a first dose of measles vaccine will respond to the second dose, although there have been recent measles outbreaks that included a substantial proportion of cases who had received two doses of measles vaccine (164).

Vaccine Side Effects

Standard doses of currently licensed measles vaccines are safe in immunocompetent children and adults. Fever to 39.4°C (103°F) occurs in 5% to 15% of seronegative vaccine recipients, which can induce seizures in children predisposed to febrile seizures, and 2% to 5% of vaccine recipients develop transient rash. Attenuated measles vaccine is not a recognized cause of SSPE, which has declined in incidence in parallel with the vaccine-induced decline in the incidence of measles. Mild transient thrombocytopenia has been reported with an incidence of approximately one in a million vaccine recipients (165).

Although assumed to be rare, the risk of disease caused by attenuated measles vaccine virus in HIV-infected persons is unknown. The only documented case of fatal disease induced by measles vaccine virus in an HIV-infected person was in a 20-year-old man who died with MeV giant cell pneumonitis 15 months after receiving his second dose of measles vaccine (166). He had a very low $CD4^+$ T-lymphocyte cell count but no HIV-related symptoms at the time of vaccination. Fatal, disseminated infection with measles vaccine virus has been reported rarely in persons with other impairments of immune function, and MIBE caused by vaccine virus was reported in a child with an uncharacterized immune deficiency (130).

As with wild-type MeV infection, measles vaccine is associated with immunosuppression. However, this immune suppression is less than after wild-type MeV infection and resolves within weeks after vaccination (167). Manifestations include decreased lymphoproliferative responses to mitogens and antigens, altered patterns of cytokine production, and suppression of delayed-type hypersensitivity skin test responses. Tuberculin skin test reactivity may be abrogated for 4 to 6 weeks after immunization, but unlike wild-type MeV infection, measles vaccine does not exacerbate tuberculosis.

Much public attention has focused on a purported association between MMR vaccine and autism following publication of a report in 1998 hypothesizing that MMR vaccine may cause a syndrome of autism and intestinal inflammation (168). The events that followed and the public concerns over the safety of MMR vaccine led to diminished vaccine coverage in the United Kingdom and provided important lessons in the misinterpretation of epidemiologic evidence and the communication of scientific results to the public (169). The publication that incited the concern was a case series describing 12 children with a regressive developmental disorder and chronic enterocolitis. Nine of the children had autism. Onset of the developmental delay was associated by the parents with MMR vaccination in eight children. This

simple temporal association was misinterpreted and misrepresented as a possible causal relationship, first by the lead author of the study and then by the media and public. Subsequently, several comprehensive reviews and additional epidemiological studies rejected evidence of a causal relationship between MMR vaccination and autism (170), and the original paper was retracted because of ethical violations and misrepresentation of data.

Contraindications

Contraindications to measles vaccination include pregnancy (a theoretical risk to the fetus); anaphylactic allergy to eggs, gelatin, or neomycin; severe immune suppression associated with HIV-infection defined as percentage of $CD4^+$ T lymphocytes < 15%, other conditions associated with severe impairment of cellular immunity, and recent administration of Ig, IVIg, or other Ig-containing products.

Investigational Approaches to Measles Vaccination

A new measles vaccine approach would be advantageous if it would allow vaccination of infants before 6 months of age. This would both close the "window of susceptibility" between decay of maternal antibody and vaccination and facilitate delivery by allowing measles vaccine to be given at the same time as other WHO EPI vaccines. Additional motivations for development of new vaccine approaches would be to increase thermostability, eliminate the use of needles and syringes for delivery, and provide a vaccine that would be safe for immunocompromised individuals (171).

A number of experimental vaccines have been developed, including individual MeV proteins (usually H and F) expressed in plants, by viral or bacterial vectors, from DNA, or as peptides or proteins. Delivery of viral genes into host cells for processing and antigen presentation without the need for virus infection, along with thermostability, inexpensive manufacture, and the potential for mucosal administration make DNA vaccines an attractive possibility for development. Studies in juvenile macaques indicated that DNA vaccines could protect from measles and were not associated with predisposition to atypical measles (94). Adjuvants improved responses and protection in macaques. In addition to DNA, several viruses and bacteria have been used to express MeV proteins and tested as experimental vaccines. These include alphavirus replicon vectors, shigella, salmonella, and Bacille-Calmette-Guerin, the mycobacteria used for neonatal immunization against tuberculosis (171–173). These vaccines have received limited testing in humans and are not currently being advanced.

Needleless respiratory delivery of the current attenuated measles vaccine has been extensively evaluated. It is postulated that this route of vaccination would be more natural, be easier to administer, generate less medical waste, and might also overcome the presence of maternal antibody to allow vaccination at an earlier age (174, 175). Reconstituted aerosolized liquid measles vaccine has been studied for more than three decades (176–178) and recently dry powder versions have also been developed. Both are safe and immunogenic in humans and macaques (179, 180). If employed for routine vaccine delivery, either the liquid nebulized or inhalable dry powder vaccine is projected to provide substantial cost savings for measles immunization through elimination of the need for waste management of used needles and improved vaccine coverage particularly during mass campaigns (181).

Measles Vaccination Strategies

Different goals for measles control have been established, necessitating different vaccination strategies. Three broad goals can be defined: mortality reduction, regional elimination, and global eradication.

Mortality Reduction

Mortality reduction, the least demanding of the three goals, calls for a reduction in measles mortality from a predetermined level through reductions in incidence, case fatality, or both (182). Although a reduction in case fatality using appropriate case management is an important component, measles mortality reduction is achieved largely through a reduction in incidence. To reduce incidence, measles vaccine is administered as a single dose through routine immunization services in child health clinics, with the optimal age of immunization determined by the transmission intensity and rate of decline of maternal antibodies. If vaccination coverage is sufficiently high, substantial reductions in incidence and mortality occur, the interepidemic period lengthens, and the age distribution shifts toward older children, further contributing to a reduction in case fatality.

Regional Elimination

Measles elimination is the interruption of MeV transmission within a defined geographic area, such as country, continent, or region. Small outbreaks of primary and secondary cases may still occur following importation from outside the region, but sustained transmission does not occur. Because of the high infectivity of MeV and the fact that not all persons develop protective immunity following vaccination, a single dose of measles vaccine does not achieve a sufficient level of population immunity to eliminate measles. A second opportunity for measles immunization is necessary to eliminate measles by providing protective immunity to children who failed to respond to the first dose and to those who were not previously vaccinated. Two broad strategies to administer the second dose have been used. In countries with sufficient infrastructure, the second dose of measles vaccine is administered through routine immunization services, typically prior to the start of school (4 to 6 years of age) or during the second year of life. High coverage levels can be ensured by school entry requirements. A second approach, first developed by the Pan American Health Organization (PAHO) for South and Central America (183), involves mass immunization campaigns (called SIAs) to deliver the second dose of measles vaccine. This strategy was very successful in eliminating measles in South and Central America and subsequently resulted in a marked reduction in measles incidence and mortality in parts of sub-Saharan Africa. This strategy has been adopted by most countries (not the United States) but is difficult to sustain in the long term because of the financial and human resources required for these large campaigns.

The PAHO strategy consisted of four subprograms: catch-up, keep-up, follow-up, and mop-up. The catch-up phase is a onetime, mass immunization campaign that targets all children within a broad age group regardless of whether they have previously had wild-type MeV infection or measles vaccination. The goal is to rapidly achieve a high level of population immunity and interrupt MeV transmission. These campaigns are conducted over a short period of time, usually over several weeks, and during a low transmission season. Under the PAHO strategy, children 9 months to 14 years of age were targeted for vaccination. In many coun-

tries, this is a substantial proportion of the total population. The appropriate target age range depends upon the age distribution of measles seropositivity. In regions with endemic measles, the majority of older children are likely to be immune. Nevertheless, seroprevalence studies usually are not conducted prior to catch-up campaigns, and this broad age range first adopted by PAHO has been widely used in sub-Saharan Africa and Asia. These campaigns require large investments of financial resources and personnel; extensive logistical planning to transport and store vaccines, maintain cold chains, and dispose of syringes and needles; and community mobilization to ensure participation. But if successful, SIAs are cost effective and can abruptly interrupt MeV transmission with dramatic declines in incidence and mortality.

Keep-up refers to the need to maintain greater than 90% routine measles vaccine coverage through improved access to measles vaccination and a reduction in missed opportunities (e.g., because of false contraindications to vaccination). Follow-up refers to periodic mass campaigns to prevent the accumulation of susceptible children. Follow-up campaigns typically target children 1 to 4 years of age, a narrower age group than targeted in catch-up campaigns. Follow-up campaigns should be conducted when the estimated number of susceptible children reaches the size of one birth cohort, generally every 3 to 5 years after the catch-up campaign. Mop-up campaigns target difficult-to-reach children in sites of measles outbreaks or low vaccine coverage. Difficult-to-reach children include those living on the street or in areas of conflict.

Global Eradication

The possibility of measles eradication has been discussed for almost 40 years (184). Serious discussion of measles eradication began in the late 1960s, when smallpox eradication was nearing completion and the effective, long-term immunity induced by measles vaccine became apparent. MeV meets many of the biologic criteria for disease eradication (185). MeV has no nonhuman reservoir and is accurately diagnosed, and measles vaccination is a highly effective intervention. Although MeV displays sufficient genetic variability to conduct molecular epidemiologic analyses, the antigenic epitopes against which protective antibodies develop have remained stable. Where MeV differs from smallpox and polioviruses is that it is more highly infectious, necessitating much higher levels of population immunity to interrupt transmission.

The vaccination strategy necessary for measles eradication is not different from that of regional elimination, only that the target population is global. The success of measles elimination in large geographic regions suggests that measles eradication is possible. Two doses of measles vaccine, administered through routine immunization services or via SIAs would need to be administered to the children of the world. Many believe this to be a realistic and morally imperative goal, but as polio eradication efforts have shown, the endgame may be full of challenges.

TREATMENT

Symptomatic and Supportive Therapy

Treatment of uncomplicated measles is symptomatic and includes bed rest, hydration, and antipyretics. Secondary bacterial infections require prompt treatment with antibiotics. Antibiotics are indicated for children with measles who have clinical evidence of bacterial infection, including pneumonia, otitis media, skin infection, eye infection, or severe mouth ulcers. *Streptococcus pneumoniae* and *Haemophilus influenza* type B were the most common causes of bacterial pneumonia following measles prior to widespread use of vaccines against these pathogens. More recent data on bacterial pathogens causing pneumonia in children with measles are lacking. Whether all children with measles or all hospitalized children with measles should be given prophylactic antibiotics remains controversial. Limited evidence suggests that antibiotics administered as prophylaxis to all children presenting with measles may reduce the incidence of pneumonia but not mortality (131). The potential benefits of antibiotic prophylaxis need to be weighed against the risks of adverse effects and accelerating antibiotic resistance.

Vitamin A

Vitamin A is effective for the treatment of measles, and its administration results in marked reductions in morbidity and mortality in hospitalized children with measles treated with vitamin A. The WHO recommends administration of two daily doses of 200,000 IU of vitamin A to all children with measles 12 months of age or older. Lower doses (100,000 IU) are recommended for children less than 12 months of age. Overall, this regimen results in a 64% reduction in the risk of mortality (186). Pneumonia-specific mortality is reduced, and the impact is greatest in children younger than 2 years of age (186). The mechanisms by which vitamin A reduces measles morbidity and mortality are not known, but these effects are likely mediated through beneficial effects on epithelial cells and host immune responses.

While vitamin A deficiency is not a recognized problem in the United States, many children in the United States with measles have low serum vitamin A levels and these children have increased morbidity following measles. The Committee on Infectious Diseases of the American Academy of Pediatrics recommends the administration of two consecutive daily doses of vitamin A (200,000 IU orally for children 1 year and older; 100,000 IU for children 6 months to 1 year of age) be considered for children 6 to 24 months of age hospitalized for measles and its complications, as well as for children older than 6 months with measles who have immunodeficiency, ophthalmologic evidence of vitamin A deficiency, impaired intestinal absorption, moderate to severe malnutrition, or recent immigration from areas where high measles mortality rates have been observed.

Vitamin A has been widely distributed through polio and measles SIAs as well as through routine child health services and fortified foods. Prophylactic vitamin A supplementation of apparently healthy children has resulted in a 39% reduction in measles-associated mortality (187).

Antiviral Therapy

There is no antiviral therapy of proven efficacy for measles. Ribavirin inhibits MeV replication in cell culture and has been reported to reduce the severity of measles in children and adults (188). Anecdotal reports have described previously healthy, pregnant, and immunocompromised patients with measles pneumonia and immunocompromised patients with subacute measles encephalitis who recovered following treatment with aerosolized and/or intravenous ribavirin (127, 129). However, the clinical benefits of ribavirin treatment have yet to be demonstrated as only a few

anecdotal cases have been reported, and its use in measles is investigational.

Numerous therapeutic agents have been used to treat SSPE, including IFN, ribavirin, amantadine, isoprinosine, inoseplex, and levamasole. Experience has been anecdotal and any benefits have been transient at best.

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Mumps Virus

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Mumps virus, a member of the paramyxovirus family, causes a distinctive and generally benign systemic infection that is clinically characterized by fever and parotitis. In older literature, mumps was often termed "epidemic parotitis." Historically, mumps occurred commonly among school-aged children but dramatically declined following the introduction of routine vaccination. Significant mumps outbreaks continue to occur, however, among previously infected or vaccinated individuals for reasons that are not fully understood. These cases of reinfection or vaccine failure can sometimes be difficult to diagnose due to mild clinical presentations and can be difficult to confirm due to limitations of current laboratory testing.

VIROLOGY

Classification

Mumps virus is a member of the order *Mononegavirales* (viruses with nonsegmented, negative-sense, single-stranded RNA genomes), family *Paramyxoviridae*, subfamily *Paramyxovirinae*, and genus *Rubulavirus*. Serologic testing by hemagglutination inhibition or by *in vitro* plaque reduction neutralization assays has identified only one serotype of mumps virus. However, strain-specific variation in cross-neutralizing antibody titers has been observed with individual sera, and minor antigenic differences among mumps virus isolates can be detected using panels of glycoprotein-specific monoclonal antibodies (1–4).

Virus Composition

Mumps virions are markedly pleomorphic, irregularly spherical particles that range from 50 to >300 nm in diameter (average about 200 nm) (5–7). An outer envelope derived from the host cell plasma membrane encloses a helically coiled ribonucleocapsid core (8). The envelope is about 10 nm thick and is composed of three layers: glycoprotein spikes that project 10 to 15 nm from the outer surface of the envelope, which consists of a lipid bilayer acquired from the host cell as the virus buds from the cytoplasmic membrane, and an inner structural matrix protein (Fig. 1) (9).

Genome

The mumps virus genome is a linear molecule of single-stranded, negative-sense RNA (approximately 15,384 bases) that serves as a template for both transcription and replication by the virus-encoded RNA polymerase. Wild-type mumps virus genomes adhere to the "rule-of-six," meaning that the total number of bases in the genome must be evenly divisible by six (10), although this requirement may not be absolute (11). The gene order is 3′-NP-V/P/I-M-F-SH-HN-L-5′ (Fig. 2). The letter designations are NP (nucleoprotein), P (phosphoprotein), V and I (no alternative names), F (fusion), SH (small hydrophobic protein), HN (hemagglutinin-neuraminidase), and L (large protein; catalytic RNA polymerase subunit). There are 3′-leader and 5′-trailer untranslated regions (UTR) at the ends of the genome that encode the genomic and antigenomic promoter sequences, and short intervening sequences between each gene that encode transcription control signals (12–14). The V, P, and I genes overlap and share 5′ mRNA coding sequence. The V gene mRNA is produced by faithful transcription of the genome, but the P and I gene transcripts contain additional nontemplated G residues that are inserted by the RNA polymerase during transcription and result in shifted open reading frames (15–17).

There are 12 known genotypes of mumps virus recognized by the World Health Organization that are distinguished by sequences of the SH and HN genes (18). The SH gene sequence is the most variable of all the mumps virus genes (up to 20% heterogeneity among genotypes). The genotypes are designated by letters A through N (excluding E and M). The global distribution and temporal circulation of mumps genotypes has been described (18), but improved molecular epidemiology is needed to strengthen surveillance efforts.

Viral Proteins

The nucleoprotein (NP) is a major viral structural protein. Monomers of NP assemble into a left-handed helical coil, forming a tubular structure that encapsidates the genomic RNA. This is referred to as the ribonucleocapsid, or ribonucleoprotein complex (RNP) (19–23). An 18-Å resolution structure of the mumps RNP has been reported based on cryoelectron microscopy (20). The RNP helps protect the genome against degradation and is essential for transcription to occur (21).

The mumps virus RNA-dependent RNA polymerase (RdRp) is composed of two subunits: the large protein (L) and the phosphoprotein (P). There are few published reports

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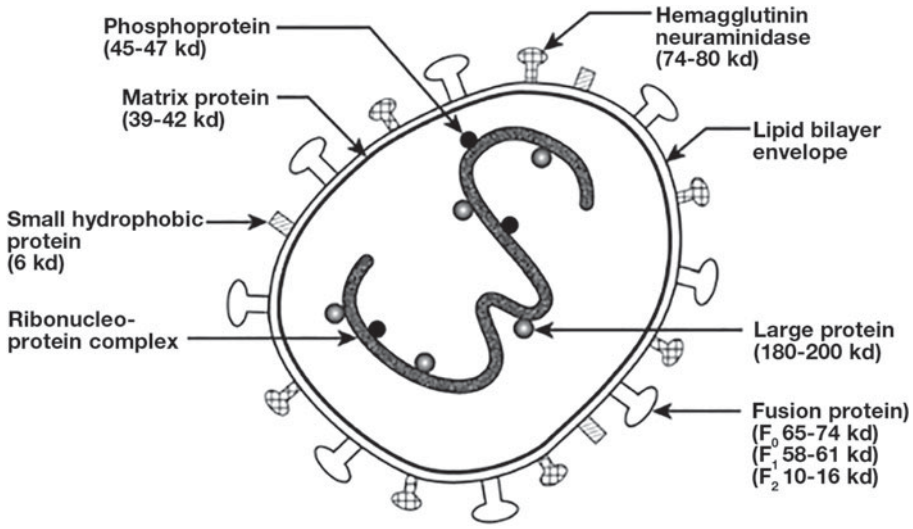

FIGURE 1 Structure of the mumps virion. Molecular weights of viral proteins as measured by gel electrophoresis are shown in parentheses. Data from Galinski and Wechsler (372).

that specifically describe the activities of mumps virus L subunit (14), but the L proteins of closely related paramyxoviruses provide catalytic activity for RNA polymerization, mRNA capping, and polyadenylation (24, 25). The phosphoprotein (P) is an essential cofactor for the polymerase that is required to uncoil the nucleocapsid to allow the L subunit access to the genomic RNA template (20, 26). Both the P and NP proteins are phosphorylated, which can alter the efficiency of RNA synthesis depending on the specific residues involved (21, 27). The kinases that phosphorylate mumps P and NP proteins are not known, and mumps virus does not encode any proteins with known kinase activity. However, based on studies of related paramyxoviruses, host cell kinases such as CKII, PKC-ζ, AKT, or PLK1 may be responsible (27–29).

The hemagglutinin-neuraminidase (HN) and fusion (F) membrane proteins have cooperative roles in virus attachment and entry into host cells (30–32). The HN protein is a type II glycoprotein; it is anchored in the plasma membrane at the N terminus, and it is glycosylated (33–35). Structurally, the HN protein forms a homo-tetramer that is characterized by a globular head sitting atop a stalk domain (36). The HN head binds to N-acetyl neuraminic (sialic) acid residues on host cells and attaches the virus particle to the cell prior to fusion (37). As a result of the sialic acid binding property, the HN protein has the ability to agglutinate red blood cells (38). The HN globular head domain also has a neuraminidase activity that cleaves sialic acid, enabling release and preventing aggregation of virus particles as they bud from the host membrane (39).

The F membrane protein is a homo-trimer (40–42). It is synthesized as an inactive precursor (F_0) that is cleaved into a biologically active form consisting of two fragments, (F_1) and (F_2), that are joined by a disulfide bond (43, 44). An irreversible change in the F protein conformation is triggered by direct interaction with the HN stalk domain. This change in structure is ATP independent and it brings F into a lower-energy state, resulting in fusion of the virus with host cell membranes (30, 36, 45–47). HN and F are essential not only for virus-cell membrane fusion, but also for cell-to-cell membrane fusion and virus spread (48). As a result, multinucleated giant cells (syncytia) can be observed in cultures of cells infected with mumps virus. However, due to variation in the activities of the HN and F proteins, the degree to which syncytia are formed is dependent on the specific virus strain (44, 48–50).

The matrix (M) protein is a membrane-associated molecular scaffold that is required along with the F and NP proteins for efficient virus particle formation and release (9, 32, 51, 52). Based on studies of related paramyxoviruses, the

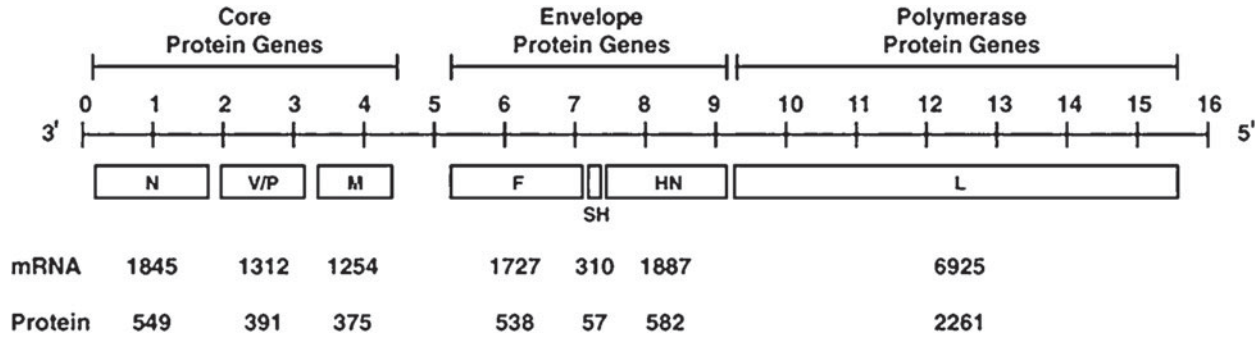

FIGURE 2 Genome organization of mumps virus. Number of nucleotides in messenger mRNA: and Protein: Amino acid residues in corresponding protein. Data from Pringle (373).

mumps M protein is thought to interact with the nucleoprotein and the tails of glycoproteins on the inside of the virion membrane to facilitate virus assembly and budding (9, 52). Mumps virus M protein also transits through the nucleus as the result of a bipartite nuclear localization sequence (NLS_{bp}) and a leucine-rich nuclear export sequence (NES). The process of nuclear transit appears to be modulated by ubiquitination and may result in interactions with components of the nuclear pore complex (52). Additional evidence indicates that M protein interactions with class E host cell proteins of the ESCRT complex are essential for virus budding (51).

Both the mumps virus V and SH proteins have been implicated as modulators of the host immune response (53–55). The V protein inhibits the expression and signaling of type I interferon, and signaling by IL-6 by inducing ubiquitin-mediated degradation of host cell STAT1 and STAT3 proteins (56–59). In addition, the V protein has been reported to inhibit the double-stranded (ds)RNA pattern recognition receptor MDA5 (60). The SH protein is only 57 amino acids long and the C terminus of SH is integrated into the viral membrane (61). Although SH is nonessential for virus growth in tissue culture, SH interferes with tumor necrosis factor (TNF)α-mediated signaling and apoptosis by unknown mechanisms (53, 62, 63). The SH protein also binds cellular ubiquilins 1 and 4, which participate in the ubiquitin-mediated proteasome degradation pathway (64). There have been reports of a protein expressed from the I gene transcript, but additional information regarding I protein function is lacking (16, 17, 65).

Replication Cycle

After binding to sialic acid on the cell surface and fusion of the virus and host membranes through cooperative interaction between the HN and F proteins, the RNP complex is released from the virus particle into the host cell cytoplasm (30). The RNP serves as a template for both transcription and replication by the viral RNA polymerase (L and P proteins) (12, 24). The polymerase engages the template at the promoter within the leader sequence at the 3′ end of the genome. As the polymerase proceeds, it initiates mRNA synthesis and releases the transcripts in response to gene-start (*gs*) and gene-end (*ge*) sequences flanking each gene (12). The mRNA transcripts are capped and polyadenylated by the virus RNA polymerase (Fig. 3) (24). The likelihood that polymerase remains engaged with the template diminishes as it continues toward the 5′ end of the genome, and termination occurs more frequently at the end of each successive gene. As a result, a gradient of mRNAs is produced, with NP and L mRNAs representing the highest and lowest abundance transcripts, respectively (24).

The molecular mechanism that determines if the RNA polymerase preferentially synthesizes monocistronic mRNA transcripts or full-length copies of the antigenome and genome is not clear. However, it is thought that the accumulation of NP protein in the cytoplasm of the infected cell drives the switch from transcription to replication (12, 22). The polymerase is stabilized by the immediate binding of NP to the nascent, newly synthesized RNA as it emerges from the polymerase complex. As a result, the polymerase does not terminate in response to gene-end signal sequences and full-length antigenomic RNA is produced. The positive-sense antigenomic RNA then serves as a template for replication of the negative-sense genomic RNA. Synthesis of the genomic RNA is initiated by a promoter located within the trailer sequence juxtaposed to the L gene at the 3′ end of the antigenome. Unlike viral mRNA, the full-length antigenomic and genomic RNA are not capped or polyadenylated (12). The mechanism that controls the relative abundance of full-length genomic and antigenomic transcripts is uncertain, but it has been suggested that binding of the V protein to L, or differential phosphorylation of the P protein might influence this process (12). The full-length negative-sense RNA copies may serve as templates for replication or as templates for secondary transcription, or they may be packaged into progeny virions (Fig. 3).

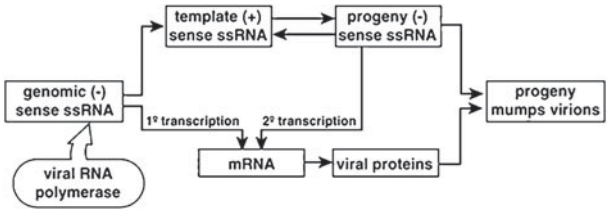

FIGURE 3 Replication strategy of mumps virus.

Assembly and budding of mumps virions from the host cell membrane is dependent upon the M, F, and NP proteins (9, 32, 51). The matrix protein orchestrates the assembly process through interactions with the ribonucleocapsid and the cytoplasmic tails of the glycoproteins along the inner surface of the cellular membrane. Evidence suggests that the functions of the M protein require ubiquitin-regulated trafficking through the nucleus and interactions with cellular class E proteins of the ESCRT complex (9, 32, 52). As the progeny virions are released from the cell by budding, they acquire a portion of the cellular cytoplasmic membrane, which becomes the virion envelope. As a result, the envelope may include some host cell membrane proteins, such as CD55 and CD46, which regulate the activity of complement (66).

Host Range

Humans are the only known natural host for mumps virus; however, a virus with approximately 90% homology to mumps virus was recently isolated from bats, suggesting the potential of this host as a reservoir (67). Experimental mumps virus infection has been attempted in a variety of animal species, but no animal models consistently reproduce the full spectrum of clinical symptoms observed in humans. Rhesus macaques appear to be the best model for mumps disease in humans, although hamsters and mice have been the preferred models for studies of neuropathology induced by mumps virus infection (68).

Cell Culture

Mumps virus can be propagated in embryonated chicken eggs (69) or in a wide variety of primate, human, and murine-derived cell lines. Vero cells (African Green Monkey kidney) are commonly used, but other cell lines that support mumps virus replication include CaCo2, PLC/PRF/5, NCI-H292, BSC-1, MCF-7, CV-1, A549, L-41, HEp2, HeLa, and CHO (66, 70–76). Although most of these cell lines have epithelial origins, there have been reports of mumps virus replication in cultures of human peripheral blood mononuclear cells, with preferential replication in T cells and T-cell-derived lymphoblastoid cell lines (77, 78). Mumps virus has rarely been isolated from blood of infected individuals, however, likely due to the development of neutralizing antibody during infection (79, 80). *In vitro* cytopathic effects may appear any time from 24 hours up to 1 week after

inoculation. A characteristic cytopathic effect of mumps infection is cell-to-cell fusion, resulting in giant multinucleated syncytia (Fig. 4). Varying degrees of fusion and plaque morphology can occur and are dependent on the virus strain and cell lines involved (81). However, the degree of virulence of mumps virus strains cannot be predicted by *in vitro* cytopathology. Persistent, generally noncytopathic mumps virus infection can be established in cultured cells, including Vero (82), L929 (83), BHK-21 (84), MCT (85), human HEp2, L-41, MCN, Lung-To cells (74, 86), and primary human cells derived from synovial membranes (87) and conjunctiva (88, 89).

Stability and Inactivation

Infectivity of mumps virus can be maintained for months to years if stored at −70°C, or if it is lyophilized and stored at 4° C (90, 91). The virus is stable within the pH range of 4.6 to 8.5 (69), but it can be inactivated by heating to ≥56°C or by exposure to formalin, organic solvents, detergents, or ultraviolet irradiation (92, 93).

EPIDEMIOLOGY

Transmission Patterns

Mumps has a worldwide distribution. In susceptible urban populations, it is primarily a disease of school-aged children, with over 50% of cases occurring between 5 and 9 years of age (94). In unvaccinated populations, over 90% of children have antibodies against mumps virus by age 15 (95). Infection is commonly acquired at school, with secondary spread to susceptible family members. Mumps is rare in children under 1 year of age, presumably due to the protective effect of passively acquired maternal antibody. Before the release of the mumps vaccine in the United States in 1967, mumps was an endemic disease with annual peak activity occurring between January and May. Localized epidemics of mumps in closed populations (isolated communities, boarding schools, military units, etc.) were also well recognized.

Transmission

Mumps virus is spread by the respiratory route and can be transmitted by contact with respiratory secretions or saliva from infected individuals (96, 97). The incubation period between infection and symptom onset is approximately 18 days, but cases may occur from 12 to 25 days after exposure (97, 98). The virus can be isolated from and spread by infected individuals with no clinically apparent symptoms (97, 99). Many studies since the 1940s have estimated that 30% or more cases of mumps virus infection may be asymptomatic (100–107). Although mumps is easily spread, it is less contagious than measles (105, 108). The basic reproduction number (R_0, the estimated number of secondary cases expected to result from a single index case in a susceptible population) for mumps has been estimated as 10 to 12, while for measles it is 15 to 17 (109, 110).

Incidence and Prevalence Epidemiology of Mumps in the United States

The largest number of mumps cases reported in the United States occurred in 1941 when the incidence was 250 cases per 100,000 persons (111). When the mumps vaccine first entered clinical usage in 1968, the incidence of mumps was 76 cases per 100,000 persons. Mumps vaccine was recommended for routine use in the United States in 1977. In 1985, only 2,982 cases of mumps were reported (1.1 cases per 100,000 population), which was a 98% reduction from the 185,691 cases reported in 1967 (112). Between 1985 and 1987, the incidence of mumps in the United States increased 5-fold to 5.2 cases per 100,000 population (111, 113). More than one-third of the cases reported during this interval occurred in adolescents, reflecting the failure to adequately vaccinate this cohort of children during the 1970s (114, 115).

In 1989, the Centers for Disease Control Advisory Committee on Immunization Practices (ACIP) issued a recommendation that all children receive a second dose of the measles-mumps-rubella (MMR) vaccine, primarily to ensure seroconversion to measles (112). Following this recommendation, the incidence of mumps in the United States also continued to decline and only 231 cases were reported in 2003 (116, 117). The importance of immunization of adolescents and young adults was further emphasized by a large outbreak (over 56,000 reported cases) of mumps in the United Kingdom in 2004 to 2005 (109, 118). Many of the cases occurred in university students who were too old to have been vaccinated, but too young to have been exposed to epidemic natural infection (119).

High two-dose MMR vaccination coverage in the United States led to the declared elimination of endemic sustained transmission of measles (120) and rubella (121) viruses in 2000 and 2004, respectively. It appeared that mumps would also soon follow suit. By testing sera from the 1999 to 2004 U.S. National Health and Nutrition Examination Survey (NHANES), the overall age-adjusted seroprevalence of IgG to mumps virus was shown to be 90% (122), a figure thought to be near the lower end of the level of seroprevalence required to achieve population immunity. In 2005 to 2006 a series of outbreaks in the United States and Canada demonstrated the potential for mumps virus to cause limited, localized outbreaks even among vaccinated populations (123, 124). In 2006, a total of 6,584 cases of mumps were reported in the United States, with a national incidence of 2.2 cases per 100,000 population (123). The American

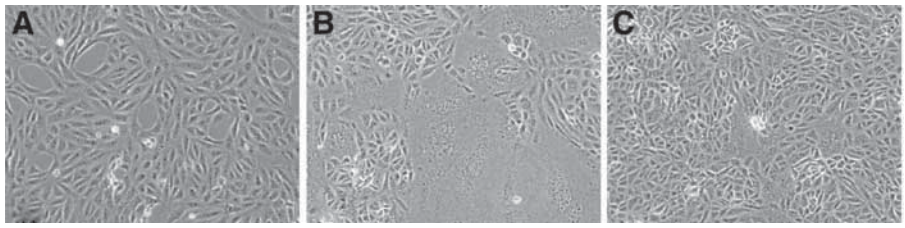

FIGURE 4 Cytopathic effect of mumps virus replication in tissue culture. Cell-to-cell fusion in Vero cells during the course of infection with mumps virus (10x magnification). (A) uninfected, and 48 hours post infection (m.o.i. = 0.01) with (B) Jeryl Lynn strain, and (C) wild-type virus (genotype G).

outbreak was centered in the Midwest and peaked in April 2006; 34% of the cases occurred in Iowa. Among the Iowa cases, 7% were unvaccinated; 14% had received one dose of MMR; 49% had received *two or more* doses of MMR; and 30% had unknown vaccine status. The highest age-specific rate was in persons aged 18 to 24 years (median age 22 years), many of whom were college students (125). Another relatively large mumps outbreak (>3,500 cases) occurred in 2009 to 2010 in New York and New Jersey, causing disease primarily among adolescent boys attending Orthodox Jewish schools (126). Mumps transmission in this highly vaccinated population was apparently facilitated by the close face-to-face interactions among the students (127, 128). Numerous other outbreaks among highly vaccinated populations have been reported from around the world (129–135).

Mortality

Mumps is generally a benign disease; the case-fatality ratio from 1966 to 1975 was 1.0 to 3.4 per 10,000 cases, with most deaths resulting from encephalitis (136). This ratio is likely an overestimation due to underreporting of mild or asymptomatic cases. No deaths were reported during mumps outbreaks in the United States from 2006 to 2010 (137).

The basis for the recurrent mumps outbreaks among vaccinated individuals is not understood and may be multifactorial, but it is not completely unexpected since wild-type mumps virus infection does not necessarily confer lifelong immunity (138, 139). Although failure to vaccinate was a problem in past decades, the vaccine coverage rate for *one or more* doses of MMR was 91.9% for American children aged 19 to 35 months in 2013 (although MMR coverage was below 90% in 17 states) (140). Primary vaccine failure due either to reduced vaccine potency or host immune factors seems unlikely (141). Secondary vaccine failure (waning immunity over time) is certainly a concern but has been at least partially addressed by the addition of a mandatory second dose of MMR (142, 143). Some investigators have suggested the possibility of infection caused by variant wild-type mumps viruses and enabled by a genotype-specific neutralizing antibody response (144). Neutralization capacity of vaccine-induced antibodies may be reduced against more antigenically divergent mumps virus strains (3). However, other studies have demonstrated that sera from vaccinated children will effectively neutralize a spectrum of genotypically diverse wild-type mumps strains, arguing against the immune escape hypothesis (145). A combination of these factors could result in suboptimal population-level immunity in high-risk settings such as schools or college campuses (146).

Some have suggested the need for an improved mumps vaccine with a longer duration of protection (147). Alternatively, the propensity for mumps to spread among vaccinated adolescents in schools and communal living environments with high-intensity exposures raises the question of whether a revised vaccination schedule with a focus on young adults should be considered (123, 134, 148–150).

PATHOGENESIS

Viral Replication

Epithelial cells of the nasal and oral mucosa and the upper respiratory tract are assumed to be the initial sites of virus replication based on historic studies of experimental human inoculation (96, 97). Virus can be isolated from saliva for approximately 5 to 6 days before and following the onset of symptoms (97, 151, 152), although the likelihood of detecting virus in the saliva rapidly diminishes by 3 days after the onset of symptom (153). As the infection progresses, the virus becomes disseminated systemically, but how this occurs is not clear since the virus has been rarely isolated from blood (79, 80, 96). Lymphocytes staining positively for the NP protein have been observed in the nasal mucosa, leptomeninges, and lungs in experimentally infected rhesus macaques (68). In addition, reports of *in vitro* infection and replication of mumps virus in human peripheral blood mononuclear cells suggest these cells may be important for spreading the virus to secondary sites of replication (77, 78). Difficulties with virus isolation from blood may be due to the development of neutralizing antibody around the time of symptom onset (79).

Based on autopsy reports, clinical observations, and experimental infection of animal models, the parotid gland, testes, kidney, and tissues of the central nervous system (CNS) are significant sites of disseminated virus replication (96, 154). Mumps virus has been detected in various human tissues and secretions depending on the timing of sample collection and severity of infection. These include the parotid gland (155), testes (156), semen (157), kidney (158), urine (159), thyroid (160), tears (161), brain (162), cerebrospinal fluid (CSF) (163, 164), myocardium (165), breast milk (166), and placenta (167). Mumps can be transmitted through the placenta to the fetus (96, 168, 169), and spontaneous abortion has been reported following maternal infection during the first trimester of pregnancy (170, 171). Although congenital malformation has been reported following mumps infection *in utero* (171), the rate of occurrence is statistically similar to uninfected controls (172).

Pathology

Since mumps is rarely a fatal illness, few pathologic descriptions from autopsy series are available and usually involve patients who died of acute encephalitis (155, 162, 173–176). A hallmark manifestation of infection is swelling of the parotid salivary gland (parotitis), which occurs in 60% to 70% of individuals depending on age and immune status of population (101, 102). Parotid gland histopathology is characterized by diffuse periductal and interstitial edema with infiltration by lymphocytes and monocytes. The ductal epithelial cells degenerate and the ductal lumen becomes occluded with neutrophils and necrotic debris, but the glandular cells are generally not involved (155, 177).

Orchitis is a common complication of mumps virus infection in postpubertal men (approximately 30% of cases) (96, 102, 178, 179). Virus has been recovered from testicular biopsy material and semen, indicating that testicular tissue is directly infected (156, 157). Histopathology demonstrates severe and sometimes hemorrhagic interstitial edema with substantial infiltration of leukocytes and separation of the seminiferous tubules (174, 179–181). Swelling within the closed space bounded by the tunica albuginea causes severe pain and can produce vascular insufficiency and areas of infarction. Necrosis of the germinal epithelium and atrophy of the testicle along with scarring and fibrosis may also occur (179, 180). Temporary reduction in the levels of testosterone and inhibin B and elevations of luteinizing hormone have been observed along with negative effects on spermatogenesis (179, 182). Absolute infertility following mumps orchitis is rare, but impaired fertility has been estimated to occur in approximately 13% of patients (179).

Mumps virus frequently infects the kidney, resulting in viruria and microscopic hematuria, with abnormal renal

function evidenced by reduced creatinine clearance (159). Renal injury is usually minimal, but infection may rarely result in severe interstitial nephritis and acute renal failure with damage to the tubular epithelium, immune-complex-mediated glomerulonephritis, and edema with infiltration of mononuclear cells (158, 183–188). Pancreatitis has been reported in approximately 2% of cases (123, 189), but direct evidence for virus isolation from the pancreas in humans is lacking. A few reported severe cases of pancreatitis during acute infection have been accompanied by hemolytic anemia and renal failure (190–193). Pancreatitis associated with mumps infection can occur in the absence of parotitis (194). Infection may also result in thyroiditis. Mumps virus has been isolated from biopsy specimens of the thyroid; histology demonstrates infiltration of leukocytes around the follicles and giant cell formation with destruction of the acinar cells (160). Although cardiac complications resulting from infection are rare, mumps virus has been detected in the myocardium of several patients with endocardial fibroelastosis (165), and infection may result in interstitial fibrosis with hypertrophy and atrophy of heart muscle fibers (195).

In the prevaccine era, mumps infection was a common cause of deafness. Histopathologic changes of the inner ear in one individual who experienced hearing loss as a result of mumps prior to death (196, 197) were limited to the cochlear duct contents and the peripheral cochlear neuron. Degeneration of the organ of Corti and the stria vascularis was observed with complete atrophy of the basal coil and substantial damage to the tectoral and Reissner's membranes.

Central Nervous System

Central nervous system involvement during mumps virus infection is common. Based on observed pleocytosis in CSF, asymptomatic meningitis has been reported in up to 55% of suspected mumps cases who have undergone lumbar puncture (198). Mumps virus has been isolated from CSF obtained from patients with meningitis without other clinical symptoms (164), and virus can be detected in CSF collected during the early phases of mumps meningoencephalitis in 30% to 50% of cases (199). Studies of infection in rodent models suggest that the virus enters the CNS via the choroid plexus and replicates in the choroidal and ependymal cells, resulting in the release of virus into the CSF (200, 201).

Brains of individuals who died with encephalitis as a result of infection have exhibited a range of neuropathologic findings including diffuse cerebral edema, meningeal infiltration with mononuclear leukocytes, perivascular cuffing with mononuclear cells, proliferation of glial cells, focal neuronal destruction, and localized demyelinization (96, 173, 175, 176, 202, 203). Histologic changes indicate both direct viral cytopathic effect and demyelinization suggestive of autoimmune postinfectious encephalopathy. In the suckling hamster model, mumps CNS infection is associated with the development of stenosis of the aqueduct of Sylvius and with granular ependymitis (204). These findings suggest a possible but unproven linkage between mumps CNS infections and aqueductal stenosis in children (205).

Mumps virus can establish persistent infection in neuronal cell lines (206) and chronic CNS infection in animal models (207), but it does not routinely cause chronic infections in humans. In patients with mumps meningitis, the persistence of leukocytes and mumps-specific immunoglobulins in the CSF for months after the acute infection suggests the possibility of ongoing antigenic stimulation from chronic mumps CNS infection (208). Anecdotal case reports of chronic mumps encephalitis have appeared, but data are insufficient to confirm a role for mumps virus as a cause of chronic CNS infection in humans (209–213).

Virulence Factors and Attenuation

Because mumps virus is highly neurotropic and infection can result in serious neurologic sequelae, substantial effort has been placed toward elucidating the roles of individual genes in neurovirulence and attenuation. Several reports have suggested that the HN gene may have an important role in neurovirulence (214–217), and the F, M, V, and SH genes also have been reported to influence attenuation (53, 59, 81, 217, 218). However, the precise roles of individual genes and their mutations in pathogenesis have been difficult to establish, in part because specific mutations only appear to have effects in a virus-strain-specific manner (96, 219, 220).

Immune Responses

During the primary immune response to wild-type mumps virus infection (in unvaccinated individuals), virus-specific IgM is detectable in serum within the first few days following symptom onset. The amount of IgM in serum peaks by 7 to 10 days post onset and may persist for up to 6 months (221, 222). Similarly, virus-specific IgA is detectable in serum within days after the onset of symptoms, persists for up to 6 months (221), and can be detected in saliva for up to 5 weeks post onset (151, 223). The IgG antibody response is detectable within the first week of symptoms and continues to rise for approximately 1 month (222). While some studies indicate overall mumps antibody levels are maintained at a steady state throughout life (224), others indicate that neutralizing antibody levels wane over time (225). IgG1 is the dominant subclass of mumps-specific IgG (226). IgG specific for the nucleoprotein has been reported to appear first, followed by production of antihemagglutinin IgG (226). In addition, the nucleoprotein appears to be the immunodominant target and is nonneutralizing (226–228), while the HN and F proteins are neutralizing targets (229). Typical neutralizing antibody titers measured by *in vitro* plaque reduction tend to be low (230) and may wane over time (225). During a secondary immune response to mumps following acute infection, IgM may be present in up to 51% of previously vaccinated individuals (153), and rises in IgG titer and avidity have been observed (231–233). The frequency of mumps-specific memory B cells in circulation is low (234, 235). However, antibody-secreting B cells can be readily detected in the circulation during the course of acute infection (235), and mumps-specific long-lived plasma cells ($CD19^-$, $CD38^{hi}$, $CD138^+$) have been isolated from the bone marrow more than 40 years after exposure (236).

The mumps-specific T-cell response has historically been characterized using antigen-induced proliferation assays (237, 238) and cytotoxicity assays (239), by detection of interferon production (240–242), and more recently by flow cytometry (243, 244). Virus-specific lymphoproliferative responses have been described following vaccination (245, 246) and can be detected up to 20 years post vaccination (243, 247). A correlation exists between histocompatibility leukocyte antigen (HLA) haplotypes and frequencies of virus-specific T cells, suggesting that the effectiveness of the immune response to mumps may be heritable (248–252). Virus-specific memory $CD4^+$ T cells have been detected in circulation at low frequency but with greater abundance in bone marrow (244). There is little or no correlation between the mumps-specific T-cell immune response and the virus-specific antibody response (239, 247, 253). Evidence of

mumps-specific T cells can be detected even in seronegative individuals, suggesting that this may be the most robust component of the mumps-specific immune response (247). Elevated frequencies of both gamma-delta T cells (254) and mumps-specific T cells with specific clonal TCR rearrangements have been described in the CSF of patients with meningitis (255–257). Recruitment of cytotoxic T lymphocytes into the CNS in patients with mumps may play a role in the immunopathologic changes observed in human brains after fatal mumps encephalitis (256).

Elevated levels of cytokines and immunoglobulins can be measured in CSF from patients with mumps meningitis. CSF interferon levels decline within a week in patients with self-limited mumps meningitis, but they remain elevated in the CSF of those patients who have more severe CNS involvement or persistent CSF pleocytosis (257). Intrathecal production of mumps-specific IgG and IgM is a common feature of mumps meningitis in children, although there is no apparent correlation between the severity of clinical meningoencephalitis and antibody titers in the CSF (258). A delayed hypersensitivity response to intradermally injected mumps antigen usually develops following clinical illness, but it is not a reliable indicator of mumps immune status (259).

Although humoral and cellular components of the immune response to infection can be measured for many years after vaccination or wild-type infection, it is not known what specific parameters of the immune response may constitute a protective level of immunity. Neutralizing antibody is assumed to be essential for protection, but no studies have been able to define a titer that reliably assures protection (178, 260). Although there is only one serotype of mumps virus (2, 145), infections have been documented in individuals who had been previously infected or vaccinated, and who (in many cases) had preexisting antibody (100, 138, 261–266).

CLINICAL MANIFESTATIONS

Primary Infection

The clinical manifestations described below refer to non-immune patients. Although breakthrough mumps virus infections occur in vaccinated individuals, the complication rates are lower and the symptoms are typically less severe (126). Following an incubation period of about 16 to 18 days (range 12 to 25 days), symptomatic mumps begins with a short, nonspecific prodromal phase characterized by fever, malaise, headache, and anorexia. Young children may initially complain of ear pain. Up to 50% of mumps virus infections may be associated with nonspecific or respiratory symptoms and 20% to 30% are asymptomatic (96, 103). Patients often develop characteristic parotid salivary gland pain and swelling (Table 1; Fig. 5). Parotitis occurs in 60% to 70% of patients, but the frequency may range from 50% to 95% depending on population characteristics such as age and immunity (178). Although parotitis is often unilateral at onset, it may progress to the contralateral side during the course of infection. Swelling of other salivary glands may also occur, but it usually does not occur without parotitis. Involvement of the submandibular glands may mimic anterior cervical adenopathy. Sublingual gland involvement is the least frequent symptom, but it may be associated with tongue swelling.

Presternal pitting edema occurs in about 5% of patients and may be secondary to lymphatic obstruction caused by enlarged salivary glands (267). Painful parotid gland swelling progresses over 2 or 3 days, lifting the ear lobe outward and obscuring the angle of the mandible, which helps distinguish parotitis from cervical adenopathy. The orifice of Stensen's duct is often erythematous and edematous. Patients complain of trismus, difficulty chewing, and difficulty speaking. Drinking citrus juice may exacerbate the parotid pain. Fever up to 40°C and parotid enlargement peak on the third day of illness, followed by defervescence and resolution of parotid pain and swelling within about 1 week. Long-term sequelae of parotitis are uncommon, although sialectasia and recurrent sialadenitis have been reported.

TABLE 1 Clinical manifestations of mumps

Common
Salivary gland enlargement (esp. parotitis)
Lymphocytic ("aseptic") meningitis
Epididymo-orchitis (postpubertal males)
Uncommon
Hearing loss
Encephalitis
Oophoritis (postpubertal females)
Mastitis (postpubertal females)
Pancreatitis
Polyarthritis
Myocarditis
Thyroiditis
Hepatitis
Thrombocytopenia
Ocular involvement
Nephritis

FIGURE 5 Child with mumps displaying diffuse lymphedema of the neck due to parotitis. (Image courtesy of CDC Public Health Image Library.)

Routine laboratory findings in patients with mumps are generally nonspecific. The peripheral leukocyte count is slightly elevated (10,000 to 12,000 cells/mm^3) with a mild lymphocytosis (30% to 40% lymphocytes). Approximately 30% of mumps patients will have an elevated serum amylase reflecting inflammation of the salivary glands or pancreas (268); these can be distinguished by isoenzyme analysis or by pancreatic lipase determination.

Central Nervous System

CNS involvement is the most common extrasalivary manifestation of mumps and occurs with sufficient frequency (10% to 30% of mumps cases) that it should be considered a part of the natural history and not a complication (96, 198, 269). For reasons that remain unexplained, mumps CNS disease in unvaccinated individuals occurs 3 to 4 times more frequently among males than among females (176, 270, 271). In unvaccinated individuals, the spectrum of CNS diseases associated with mumps ranges from mild aseptic meningitis, which is very common, to fulminant and potentially fatal encephalitis, which is very rare, occurring in less than 0.1% of cases of acute mumps (270, 271). CSF pleocytosis is present in 40% to 60% of patients with acute mumps, although only 10% to 30% of mumps patients will have clinical evidence of meningeal irritation (Table 2). Thus, half of the mumps patients demonstrated to have CSF pleocytosis will not have CNS symptoms (176, 198).

CNS symptoms usually appear about 5 days after the onset of parotitis, although development of CNS findings before or simultaneously with parotitis is well recognized (270–272). Mumps CNS disease can also occur in patients without clinical evidence of parotitis; indeed 40% to 50% of patients with symptomatic mumps meningitis have no evidence of salivary gland enlargement. The diagnosis of mumps cannot be excluded in a patient with meningoencephalitis simply because the patient does not have clinically apparent salivary gland involvement.

Mumps CNS infection presents with high fever, vomiting, and headache that lasts for 48 to 96 hours (272–274) (Table 3). The majority of mumps patients with CNS involvement will have signs of meningeal irritation, but no evidence of cortical dysfunction. Defervescence is accompanied by overall clinical improvement; the total duration of illness in uncomplicated cases is 7 to 10 days. Mumps meningitis is a benign disease with essentially no risk of mortality or long-term morbidity.

The onset of seizures, altered level of consciousness, or focal neurologic abnormalities in a patient with mumps is indicative of encephalitis (270, 271). The mortality rate for patients with mumps encephalitis is less than 1.5% and permanent sequelae are rare. Even among patients who are profoundly encephalopathic, the probability of complete recovery is high; sustained seizures and focal neurologic deficits may indicate a less favorable prognosis (270).

TABLE 2 Initial CSF findings in patients with CNS mumps infection[a]

	Mean	Typical Range
WBCs	450/mm^3	100–1,000/mm^3
WBC differential	90% lymphocytes	70%–100% lymphocytes
Protein	65 mg/dl	30–150 mg/dl
Glucose	55 mg/dl	30–70 mg/dl

[a]Compiled from 116 cases (274, 276, 370, 371). Modified from Gnann (269), with permission.

TABLE 3 Initial clinical findings in patients with CNS mumps infection[a]

Signs and Symptoms	% of Patients
Fever	95
Vomiting	75
Headache	70
Neck stiffness	70
Parotitis	50
Lethargy	40
Abdominal pain	15
Seizures	15

[a]Compiled from 248 cases (199, 271–273). Modified from Gnann (269), with permission.

CSF pleocytosis (>5 white blood cells [WBCs]/mm^3) occurs in 40% to 60% of unvaccinated patients with mumps parotitis and is a prominent feature of patients with mumps meningoencephalitis (275) (Table 2). Lumbar puncture in patients with mumps meningoencephalitis usually reveals a normal opening pressure and an elevated leukocyte count of 200 to 600 cells/mm^3 (ranging up to 2,000 cells/mm^3) that is >80% lymphocytes (176, 271, 276). About 50% of patients will have a moderately elevated CSF protein and 10% to 20% will have moderate hypoglycorrhachia (CSF glucose 20 to 40 mg/dl) (276, 277). Depressed CSF glucose, an unusual finding in viral meningitis, has been reported most often with mumps virus, lymphocytic choriomeningitis virus, and herpes simplex virus infections. CSF pleocytosis often persists for weeks after resolution of clinical mumps disease (268). There is no definite correlation between the magnitude of the CSF abnormalities and the clinical course, although one study reported that higher CSF protein and lower CSF glucose levels were associated with longer durations of hospitalization (271, 278). Mumps virus can be isolated from about 33% of CSF specimens in which there is pleocytosis.

Numerous neurologic complications have been described in association with mumps encephalitis, including behavioral disturbances and personality changes (270, 279), seizure disorders (274), cranial nerve palsies (especially facial and ocular palsies) (280), muscular weakness (including hemiparesis), ataxia (270, 281), myelitis (282, 283), Guillain-Barré syndrome (284), and hydrocephalus (285–287).

Electroencephalograms recorded in patients with acute mumps encephalitis characteristically show moderate to severe slowing without spikes or lateralizing signs. Modern imaging techniques (computed tomography, magnetic resonance imaging, etc.) have not been systematically evaluated as diagnostic tools for patients with mumps CNS infection (288).

Special Populations

Maternal mumps infection during the first trimester of pregnancy may result in an increased frequency of spontaneous abortion; infection during the second or third trimester is generally uncomplicated (289). Fetal wastage occurs as a result of infection of the placenta and/or fetus during the early viremic phase. Villous necrosis with intracytoplasmic inclusion bodies in decidual cells has been seen in placentas from spontaneous abortions following maternal mumps (169). In addition, mumps virus has been isolated from fetal tissue following a spontaneous first trimester abortion that occurred during acute maternal mumps (168).

A possible association between mumps occurring during the first trimester and low birth weight has been described. There is no clear connection between mumps occurring during pregnancy and congenital defects (172). Mumps virus is excreted in breast milk, but perinatal mumps infection is extremely rare.

There are few reports of wild-type mumps virus infection in individuals with congenital or acquired immunodeficiency. There are two case reports of mumps leading to fatal encephalomyelitis in individuals with severe combined immunodeficiency (290), and one renal transplant patient developed acute irreversible transplant failure due to interstitial nephritis caused by mumps (291). Reports of disease caused by wild-type mumps virus among HIV-infected individuals are lacking.

Complications of Mumps

Mumps can result in a variety of complications, most of which have become much less common in the postvaccine era (137) (Table 1). Gonadal involvement can occur in both men and women with mumps (292). Epididymo-orchitis is rare in prepubertal boys with mumps, but was reported in 25% to 38% of men in the prevaccine era (293, 294). Orchitis can occur in vaccinated males who develop mumps but is less frequent and less severe (126, 295). Orchitis is usually unilateral; bilateral involvement occurs in 17% to 38% of cases. Orchitis typically develops within 4 to 10 days after the onset of parotitis, although it can develop prior to or even in the absence of parotitis (296, 297). Patients with mumps orchitis present with severe testicular pain and swelling accompanied by high fever (39°C to 41°C), nausea, vomiting, and headache (179). Physical examination demonstrates warmth and erythema of the scrotum with marked tenderness of the testis, which may be swollen to 3 or 4 times its normal size. Epididymitis is also present in 85% of cases (298, 299). The testicular swelling and constitutional symptoms resolve within 5 to 7 days, although residual testicular tenderness persists for several weeks in up to 20% of patients. Testicular atrophy may follow orchitis in 35% to 50% of cases, but impotence or sterility is uncommon even among patients with bilateral orchitis (300). A proposed association between mumps-related testicular atrophy and subsequent testicular malignancy is unsubstantiated (301).

Oophoritis occurs in 5% of postpubertal women with mumps. Women with mumps oophoritis typically report fever, nausea and vomiting, and adnexal pain. Sequelae are uncommon, although impaired fertility and premature menopause following mumps can occur. Fifteen percent of postpubertal women with mumps complain of breast swelling and tenderness consistent with mastitis.

Mumps-associated pancreatitis occurs in about 4% of cases and presents with fever, nausea, vomiting, and epigastric pain. Some epidemiologic data suggested an association between mumps pancreatitis and juvenile diabetes mellitus; however, the dramatic decline in the incidence of mumps has not been mirrored by a corresponding decline in the occurrence of juvenile-onset diabetes mellitus.

Migratory polyarthritis, or less frequently monoarticular arthritis, has been observed in mumps patients (302, 303). The pathogenesis of mumps-related arthritis is uncertain; virus has not been isolated from joint fluid, and there is no evidence of immune complex deposition. The arthritis may involve large and small joints and usually begins 10 to 14 days after the onset of parotitis. The joint symptoms may last 4 to 6 weeks, but usually resolve with no permanent joint damage.

Symptomatic mumps myocarditis is rare, although electrocardiographic (ECG) changes have been reported in 3% to 15% of patients (275). The most common ECG abnormalities are prolongation of the PR interval, flattening or inversion of T waves, and depression of ST segments. Rare cases of inflammatory myocarditis with lymphocytic infiltration in patients with mumps have been described. A proposed linkage of mumps with pediatric endocardial fibroelastosis remains unproven (165).

Sensorineural hearing loss is a well-recognized complication of mumps that occurs with a frequency of 0.5 to 5.0 episodes/100,000 mumps cases (304–307). In one series collected among military personnel, transient high frequency hearing loss occurred in 4.4% of cases (308). The onset of deafness may be either gradual or abrupt and may be accompanied by vertigo. Hearing changes result from direct damage to the cochlea by the mumps virus and may be either transient or permanent (309).

Other infrequent complications of mumps include thyroiditis, hepatitis, nephritis (184), and thrombocytopenia. Rare ocular complications of mumps include iritis, keratitis, and central retinal vein occlusion (310, 311). A mumps-virus-associated hemophagocytic syndrome has been described (312).

Clinical Diagnosis of Mumps

The presentation of a febrile child with parotitis strongly suggests the diagnosis of mumps, particularly if the individual is known to be susceptible and has been exposed to mumps during the preceding 2 to 3 weeks (154). However, the etiology of aseptic meningitis may not be apparent if there is no concomitant salivary gland enlargement, and specific laboratory testing is required to establish the diagnosis.

A variety of other infectious and noninfectious disorders can cause parotid enlargement that may be confused with mumps (313, 314). Other viruses (including parainfluenza virus, coxsackievirus, adenovirus, EBV, HHV-6, and influenza A virus) can cause fever and parotid gland enlargement (315–317). Accurate diagnosis requires viral culture, molecular diagnostics, or specific serologic testing. Parotid gland enlargement has also been described in patients with AIDS, especially children (318, 319). Bacterial parotitis (usually caused by *Staphylococcus aureus* or Gram-negative bacilli) is most often unilateral and occurs in debilitated patients with poor oral intake, postoperative patients, and premature infants. Physical examination reveals erythematous skin overlying a hard, warm, and very tender parotid gland. Parotid massage expresses pus from Stenson's duct. Tumors, cysts, and duct obstruction due to salivary stones can also result in unilateral parotid swelling.

Drugs and systemic illness can result in parotid swelling, which is typically bilateral and nontender (320). Medications associated with salivary gland enlargement include iodides, phenothiazines, phenylbutazone, didanosine, and thiouracil. Chronic diseases in which parotitis can appear include cirrhosis, diabetes mellitus, malnutrition, chronic renal failure, sarcoidosis (uveoparotid fever), tuberculosis, lymphoma, amyloidosis, and Sjögren syndrome.

LABORATORY DIAGNOSIS

The role of the laboratory in confirming clinically suspected cases of mumps infection is important because hallmark clinical symptoms like parotitis may not be present in all cases (321–324), vaccinated individuals may have mild or

modified symptoms (126), and parotitis can be caused by a number of other etiologies (316).

As described below, acute mumps infection can be confirmed by direct detection of virus in clinical samples by reverse transcription (RT)-PCR or culture and detection of virus-specific IgM in serum. These tests are available through some commercial diagnostic laboratories, many state public health laboratories, a network of regional reference laboratories designated by the Association of Public Health Laboratories, and through services offered by the federal Centers for Disease Control and Prevention. Among previously vaccinated persons, negative laboratory test results cannot be used to rule out the possibility of mumps virus infection, but positive results are useful for confirming clinically suspected cases of infection. Importantly, cases of mumps virus infection must be reported to local or state public health authorities as part of the National Notifiable Diseases Surveillance System. Additional information regarding laboratory testing, protocols, collection and transport of specimens, vaccination recommendations, answers to frequently asked questions, and further guidance for health care providers can be found at http://www.cdc.gov/mumps/index.html.

Virus Isolation and Nucleic Acid Amplification

Detection of viral RNA in clinical samples by RT-PCR is a sensitive, definitive, and preferred diagnostic method. The SH and NP gene sequences are common amplification targets, although methods targeting the F, M, and HN genes have also been described (153, 325–330). Because the SH gene sequence has the most diversity among virus strains and is used for genotype determination, it is an important amplification target for enhancing epidemiologic investigation (18). However, the NP gene sequence provides somewhat greater sensitivity of detection (153).

Virus is shed into the saliva for up to 1 week following the onset of symptoms, but it rapidly diminishes around 3 days post onset (153). To increase the likelihood of detection, oral fluid samples should be collected as soon as symptoms appear. Following thorough massage (30 seconds or longer) of the affected parotid gland, samples of oral fluid should be collected by swabbing the area around Stenson's duct with a flocked swab made of synthetic material, such as polyester, but not cotton, which may interfere with nucleic acid amplification. To maintain viability, samples should be placed in 1 to 2 ml of viral transport medium such as Dulbecco's modified Eagle's medium, Hank's balanced salt solution, or phosphate-buffered saline containing a source of protein such as 2% fetal bovine serum, 1% bovine serum albumin, or 0.5% gelatin. Samples should be maintained at 4°C if testing is to be performed within 24 hours, or frozen at −70°C if testing is to be delayed. Although oral specimens are preferred, virus may also be isolated from urine for up to 2 weeks following infection (331) or from CSF (163, 164). If viral RNA cannot be amplified directly from a clinical specimen, the specimen may be used to inoculate a cultured cell line (typically Vero cells). Passage in cell culture may allow the virus to replicate to a level that can be detected by RT-PCR and confirmed by sequencing.

Serologic Assays

Diagnosis of acute primary infection can also be reliably confirmed by the appearance of mumps-specific IgM in serum by ELISA (178, 222). In contrast, ≥50% of individuals with previous exposure to mumps virus either through wild-type infection or vaccination may not develop detectable IgM (178). The most reliable method of IgM detection is capture ELISA. Indirect ELISA and immunofluorescence assays are less sensitive or have higher rates of false-positive results (153, 178, 332). The likelihood of obtaining an IgM-positive serum sample is lower at the time of symptom onset and increases up to the fifth day following onset (153). If an acute phase serum sample collected ≤3 days after the onset of parotitis is IgM negative, a second sample collected 5 to 7 days after symptom onset should also be tested since the IgM response may require more time to develop.

The presence of virus-specific IgG in the serum is evidence of a previous immune exposure and is routinely measured by commercially available ELISA or bead-based assays (333, 334). Avidity testing of IgG may be useful for distinguishing between cases of primary and secondary vaccine failure (232, 233). *In vitro* plaque reduction neutralization tests have been used as a measure of neutralizing antibody, but they are labor intensive (335). Other historical methods used to detect mumps virus or virus-specific antibody included hemagglutination-inhibition assays (336), complement fixation (337), and immunofluorescence (338). Although humoral immune responses to mumps infection or immunization can be measured by a variety of techniques, each is limited to some extent by antigenic cross-reactivity between mumps and other paramyxoviruses (339–341).

PREVENTION

Current guidelines recommend the isolation of individuals with mumps for 5 days after the onset of parotitis, although it is recognized that viral shedding may also occur for a few days before the onset of symptoms (342, 343). Hospitalized patients with mumps should be isolated using standard and droplet isolation precautions for 5 days after onset of parotitis, the period of heaviest viral shedding (342).

Vaccines

Mumps can be most efficiently prevented by use of an effective vaccine containing live attenuated virus. A formalin-inactivated mumps virus vaccine was also developed, but the duration of protection was shorter, so the killed virus vaccine is no longer used (178). The vaccine currently used in the United States contains replication competent genotype A mumps virus attenuated by serial passage in embryonated hens' eggs and chick embryo cell cultures. The "Jeryl Lynn" vaccine contains two virus strains JL5 and JL2, which differ by >400 nucleotides (344, 345).

The mumps vaccine is a component of the MMR vaccine and is given by subcutaneous injection in two doses. ACIP recommends that the first dose of MMR be given at 12 to 15 months of age and the second dose given at 4 to 6 years of age (346). A quadrivalent vaccine (MMR plus varicella [MMRV]) is approved for use in the United States (347). Because of a very small increased risk of febrile seizures associated with MMRV vaccine use in children aged 12 to 47 months, some authorities recommend that MMR and varicella vaccines be administered separately for the first dose; the MMRV vaccine is appropriate for the second dose (137).

Mumps-specific antibody appears within 2 weeks of immunization and is eventually detectable in about 94% of children vaccinated with Jeryl Lynn MMR (348). Although antibody titers decline over time, 74% to 95% of vaccine recipients followed for 12 years still had detectable antibody following two doses of vaccine (150, 225). Mumps-specific cell-mediated immune responses appear to persist even longer (253). While mumps-specific IgG is a simple measure

of vaccine response, it is not a definitive correlate of protection from mumps virus infection (349).

Early studies optimistically estimated that a single vaccine injection provided protective immunity to 97% of recipients (350). However, studies conducted during subsequent mumps virus outbreaks indicated an efficacy rate of 75% to 91% following a single dose (115, 351). The efficacy of at least one dose of Jeryl Lynn vaccine for preventing clinical mumps is estimated to be 69% to 81% (352); the median efficacy for two doses is higher at 86% to 88% (178, 353).

The two-dose MMR vaccination schedule was adopted in the United States in 1989 in response to increased rates of measles, but it also effectively addressed the problem of primary mumps vaccine failure seen with a single dose (112). Most states now require presumptive evidence of mumps immunity (documented age-appropriate vaccination, laboratory-demonstrated seropositivity, or laboratory confirmation of disease) before children are allowed to enroll in school. Health care workers without evidence of mumps antibody should also be immunized (137).

The Jeryl-Lynn-based mumps vaccine is safe, well tolerated, and cost-effective (354–356). In healthy children, immunization usually causes no symptoms other than localized injection site reactions. However, fever, febrile seizures, rash, and parotitis are occasionally reported (137, 357). Administration of MMR is contraindicated in pregnant women, but it can be safely administered to children who are household contacts of pregnant women. MMR is also contraindicated in (i) persons with primary or acquired humoral or cellular immunodeficiency syndromes; (ii) persons with lymphoproliferative malignancies; and (iii) persons receiving systemic immunosuppressive therapy (137). MMR is recommended for HIV-infected children without evidence of severe immunodeficiency, as determined by appropriate age-specific CD4 lymphocyte counts (137, 358).

Mumps vaccine is produced in chick embryo cell cultures and may contain trace amounts of neomycin, so immunization is not recommended for any person with a history of anaphylactic reactions to that antibiotic. Although MMR may contain trace amounts of egg protein, serious allergic reactions are extremely rare, even among children with a documented egg allergy. Therefore, history of egg allergy is not considered a contraindication to MMR administration (137).

Other strains of live attenuated mumps vaccine (including Urabe AM9 and Leningrad-3) have been associated with higher rates of vaccine-associated aseptic meningitis and are no longer widely used (359, 360). In some countries, the Urabe mumps vaccine strain was replaced by the highly attenuated Rubini strain, which provides unacceptably low levels of clinical protection (139, 361). An important advance has been the development of a rat-based neurovirulence assay to assess the safety of candidate mumps vaccine viruses (218, 362, 363).

Postexposure Prophylaxis

Postexposure vaccination of mumps susceptible individuals may not provide protection or alter the course of the infection (137, 364). Mumps immune globulin is of no proven value in this setting and is not commercially available. The mumps antibody status of the exposed individual can be rapidly determined by ELISA, although it is generally safe to assume that adults born in the United States before 1957 have been naturally infected and are considered immune (112). Administration of a third dose of MMR as postexposure prophylaxis during a mumps outbreak appears safe, but the efficacy has not been established (355, 365, 366).

TREATMENT

Therapy for uncomplicated mumps consists of conservative measures to provide symptomatic relief, such as analgesics, antipyretics, rest, and hydration. There is currently no established role for corticosteroids, antiviral chemotherapy, or passive immunotherapy in mumps. Case reports and small series have claimed that administration of interferon-alpha2β is beneficial in men with mumps orchitis, but this treatment has not been adequately studied in a controlled fashion (179, 367, 368). Symptomatic measures to alleviate the pain and swelling of mumps orchitis include bed rest, scrotal support, opioid analgesics, and application of ice packs. Surgical decompression was frequently performed in the past for mumps orchitis, but is no longer recommended (369).

Patients with mumps and clinical evidence of encephalitis (e.g., altered mental status, seizures, or focal neurologic findings) should be hospitalized for observation. Supportive care for patients with mumps meningoencephalitis includes bed rest, fever control, hydration, antiemetics, and anticonvulsants as required. Lumbar puncture may temporarily relieve the headache in some patients with mumps meningitis. Corticosteroids have been used in the treatment of mumps encephalitis (274) and orchitis, but there are no data from controlled studies to support this approach, and corticosteriod use is not routinely recommended. Benefit from intravenous Ig infusion for mumps-related complications (e.g., transverse myelitis, immune thrombocytopenic purpura, acute disseminated encephalomyelitis) has been claimed in case reports, but this intervention has not been systematically evaluated and is not recommended (137, 154).

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Zoonotic Paramyxoviruses

DANIELLE E. ANDERSON AND LIN-FA WANG

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This chapter focuses on emergent paramyxoviruses that are associated with zoonotic disease. Hendra virus (HeV), Nipah virus (NiV), Menangle virus (MenPV), and Sosuga virus (SosPV) are known to have caused severe zoonotic infections, and Tioman virus (TioPV), Achimota virus (AchPV), and Mojiang virus (MojPV) are also suspected of causing them. These viruses, which have emerged or been detected over the last two decades, are potential threats to both livestock animals and humans (Table 1). In particular, HeV and NiV have caused fatal diseases in animals and humans, and outbreaks of NiV continue to occur almost annually. Molecular biological studies have made substantial contributions to the characterization of emergent zoonotic paramyxoviruses. Sequencing studies provide an accurate picture of the relative taxonomic position of these viruses and provide rapid diagnostic capabilities. In the case of outbreaks of NiV in Malaysia, Bangladesh, and India, molecular biological data quickly identified the etiologic agent present, and reverse transcriptase PCR (RT-PCR) and serologic assays were used to rapidly confirm NiV infections in humans and animals (1–4).

Other paramyxoviruses that have been documented to cause rare human infections include MenPV (5–7) SosPV (8), and TioPV (9–11). Most of these viruses share a common reservoir in large fruit bats (in the genus *Pteropus*), also known as flying foxes (12). Because of their clear potential to cause severe disease in humans and animals, NiV and HeV have been designated class C select agents and have been the focus of intense study since their emergence (13–15). Although yet to be conclusively linked to zoonotic disease, bats are suspected to also play a role in the transmission of AchPV and MojPV to humans (16). MojPV is a novel henipa-like virus detected in rats (*Rattus flavipectus*) in a cave in Yunnan Province, China, in 2012 (17). Although isolated from rats, microbats (*Rhinolophus ferrumequinum*) also reside in this cave, so it is possible that the disease observed in the three humans may represent a spillover event from either rats or bats.

VIROLOGY

Classification

HeV was originally named equine morbillivirus because initial sequencing and morphological studies conducted following its emergence in 1994 led to the conclusion that HeV was most closely related to the morbilliviruses (18). However, subsequent studies showed that HeV is neither a morbillivirus nor an equine virus. Analysis of the sequences of the entire genomes of both HeV and NiV now groups these viruses together in the genus *Henipavirus*, within the subfamily *Paramyxovirinae* (Fig. 1) (15). HeV and NiV are related viruses that share 68 to 92% amino-acid-sequence identity in their protein coding regions and 40 to 67% nucleotide-sequence identity in the untranslated regions of their genomes (3, 19). Among the other seven genera within the *Paramyxovirinae*, the henipaviruses are more closely related to the respiroviruses and morbilliviruses.

Based on the genome structure and phylogenetic analysis, both MenPV and TioPV have been tentatively classified as members of the genus *Rubulavirus* (20). AchPV1 and 2 are distinct paramyxoviruses and, although not closely related, cluster with the other bat-borne rubula-like viruses (16). The genome organization of SosPV is typical of the rubulaviruses, and phylogenetic analysis clearly demonstrated that SosPV also clusters with the bat-borne rubula-like viruses (8).

Sequencing of a number of novel paramyxoviruses has substantially increased our appreciation of the diversity within this viral family (6, 8, 9, 16, 17, 18, 21, 22). Several of these viruses demonstrate a departure from the restricted host range that has been the norm for most members of the *Paramyxoviridae*. For example, viruses closely related to the morbillivirus canine distemper virus have been associated with disease outbreaks in harbor seals (23), striped dolphins (24), and Serengeti lions (25).

Genome Structure and Gene Function

Paramyxoviruses contain a negative-sense, nonsegmented, single-stranded RNA genome ranging from 15,178 to 19,212 nucleotides in length. The genome structure is conserved with the nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), attachment (HN/H/G), and large (L) genes present in all viruses (Fig. 2).

The genomes of HeV and NiV are 18,234 and 18,246 nucleotides in length, respectively, and, until the characterization of Beilong (26) and J viruses (27), these were the largest genomes among the paramyxoviruses. The newly-characterized genome of henipa-like MojPV is also large, at 18,406 nucleotides. In contrast, the average genome size

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TABLE 1 Characteristics of zoonotic paramyxovirus infections of humans

Virus discovery and reservoir			Nonhuman species infected		Incidence and clinical disease in humans	
Virus	Year identified	Presumed Reservoir	Natural	Experimental	Human cases (fatal)	Clinical disease in humans
HeV	1994	Fruit bats	Horse, dog	Horse, cat, dog, guinea pig, ferret, bat	7 (4)	Influenza-like illness, pneumonia, encephalitis
MenPV	1997	Fruit bats	Pig	Pig	2 (0)	Febrile, rash illness
NiV	1999	Fruit bats	Pig, cat, dog, horse	Pig, cat, hamster, bat, guinea pig, squirrel monkey, ferret	557 (320)[a]	Pneumonia, encephalitis
SosPV	2012	Fruit bats	Unknown	Not done	1 (0)	Febrile, rash illness

[a]Includes one outbreak in Malaysia and Singapore (2), one outbreak in India (1), and outbreaks in Bangladesh, 2001 to 2005 (Institute of Epidemiology, Disease Control and Research Bangladesh situation reports: http://www.iedcr.org/); not all cases were confirmed by laboratory testing.

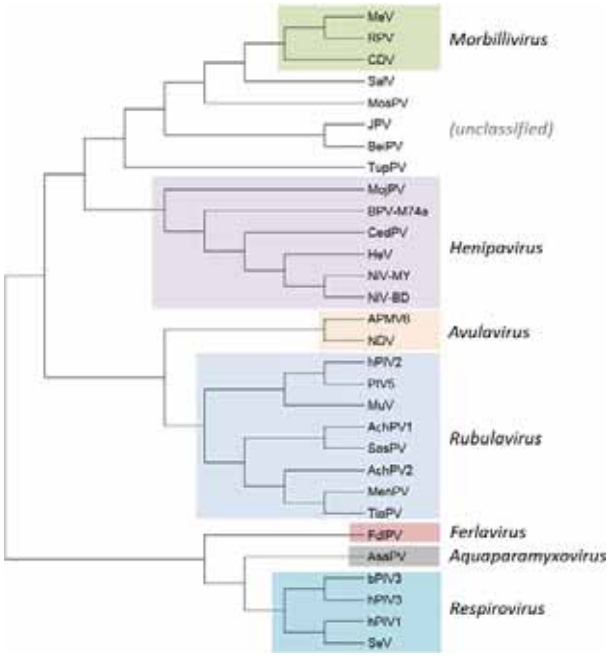

FIGURE 1 Phylogenetic analysis of the sequences of the open reading frame of the N protein gene from selected viruses in the subfamily *Paramyxovirinae*. The genus name is on the right. Abbreviations of virus names and sequence accession numbers are as follows: Achimota virus 1 (AchPV1) JX051319; Achimota virus 2 (AchPV2) JX051320; Atlantic salmon paramyxovirus (AsaPV) EU156171; Avian paramyxovirus 6 (APMV6) AY029299; Bat paramyxovirus/Eid hel/GH-M74a/GHA/2009 (BPV-M74a) HQ660129; Beilong virus (BeiPV) DQ100461; Bovine parainfluenza virus 3 (bPIV3) AF178654; Canine distemper virus (CDV) AF014953; Cedar virus (CedPV) JQ001776; Fer-de-lance paramyxovirus (FdlPV) NC_005084; Hendra virus (HeV) AF017149; Human parainfluenza virus 2 (hPIV2) AF533010; Human parainfluenza virus 3 (hPIV3) Z11575; J virus (JPV) AY900001; Menangle virus (MenPV) AF326114; Measles virus (MeV) AB016162; Mojiang virus (MojPV) KF278639; Mossman virus (MosPV) AY286409; Mumps virus (MuV) AB000388; Newcastle disease virus (NDV) AF077761; Nipah virus, Bangladesh strain (NiV-BD) AY988601; Nipah virus, Malaysian strain (NiV-MY) AJ627196; Parainfluenza virus 5 (PIV5) AF052755; Rinderpest virus (RPV) Z30697; Salem virus (SalPV), AF237881; Sendai virus (SeV) M19661; Sosuga virus (SosPV) KF774436; Tioman virus (TioPV) AF298895; Tupaia paramyxovirus (TupPV) AF079780.

for the other members of *Paramyxovirinae* is approximately 15,500 nucleotides. The genomes of the rubulaviruses MenPV (15,516), TioPV (15,522), SosPV (15,480), AchPV1 (15,624), and AchPV2 (15,504) are all typical in length. The larger size of the HeV and NiV genomes is mostly due to the unusually large sizes of the open reading frame (ORF) for the phosphoprotein (P protein) gene and the large 3' untranslated regions of several of the genes (3). The "rule of six" states that the total length of the genomic RNA of viruses within the subfamily *Paramyxovirinae* must be evenly divisible by six in order for the viruses to efficiently replicate (28). Studies with a minigenome replication assay confirmed that NiV and HeV conform to the rule of six (29).

The complete genome sequence of a horse isolate of HeV was published in 2000 (30, 31), and subsequent sequencing of other HeV isolates allowed for variants of HeV to be differentiated through analysis of the hypervariable region of the genome, despite the overall genome displaying genetic stability (32, 33). The complete genomic sequences of the NiV strains associated with the outbreaks in Malaysia in 1999 (NiV-MY) and Bangladesh in 2004 (NiV-BD) have been determined (4). The genome of NiV-BD is six nucleotides longer than that of NiV-MY, the prototype strain of NiV, and two nucleotides shorter than that of HeV. The additional six nucleotides map to the 5' untranslated region of the F protein gene. The gene order and sizes of all the open reading frames except V are conserved between NiV-BD and NiV-MY. The overall nucleotide homology between the genomes of NiV-BD and NiV-MY is 91.8%; the predicted amino acid homologies between the proteins expressed by NiV-MY and NiV-BD are all greater than 92% (4). The minor degrees of sequence variation among strains appear to be related to their geographic distribution. The N protein gene sequence of a bat isolate of NiV from Cambodia is more closely related to the sequence of NiV-MY than to the sequence of NiV-BD, while sequences obtained from samples from an outbreak in India were more closely related to NiV-BD (1). Full-genome sequence analysis of MojPV demonstrated that the closest relatives are the henipaviruses, but amino acid sequence identity only ranged from 38 to 63% compared to HeV and NiV (17).

The genomes of paramyxoviruses contain a number of conserved cis-acting signals that regulate gene expression and replication. The cis-acting signals include the gene transcription start sites, gene stop sites, RNA editing sites, genomic termini, and intergenic sequences (34). The 5' UTR contains a gene start (GS) sequence utilized for transcription initiation, and the 3' UTR contains a gene end (GE) region responsible for transcription termination. The GE sequence

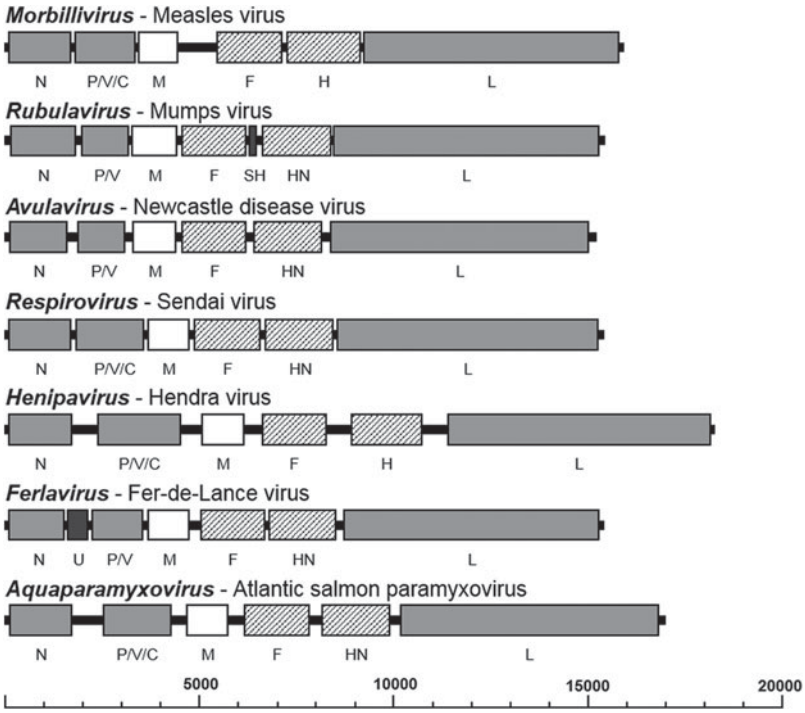

FIGURE 2 Schematic representation of the genomes of viruses in the subfamily *Paramyxovirinae*. Genomes are single-stranded, negative-sense RNA shown in the 3'-to-5' (left-to-right) orientation. Boxes indicate protein-coding regions, and solid lines indicate noncoding regions. The genome, coding regions, and untranslated regions are drawn to scale. The six conserved genes present in all paramyxovirus genomes are indicated as follows: light shaded = RNA polymerase and nucleocapsid genes (N, P and L); slanted = envelope membrane protein genes (F and attachment protein); white = matrix protein (M). The dark shaded boxes represent genes which are not commonly shared among members of the subfamily. The scale at the bottom represents genome size in nucleotides.

contains a stretch of four to seven uridine residues that act as a template for polyadenylation of the mRNA. The intergenic region is three nucleotides in length for most *Paramyxovirinae* members with the exception of rubulaviruses and avulaviruses, which can vary in length from 1 to 124 nucleotides (35, 36). However, HeV and NiV are the only paramyxoviruses in which the intergenic sequence, CTT, is conserved at every gene junction (3, 31).

Proteins

The P protein is an essential component of the replication complex for all paramyxoviruses. The P protein of NiV contains binding domains for the N protein at both its amino and carboxyl termini (37). The coding strategy for the P protein gene of the henipaviruses, respiroviruses, and morbilliviruses is that a faithful transcript of the P protein gene codes for the P protein, while the transcript encoding the V protein is produced by RNA editing. RNA editing refers to the insertion of nontemplated guanosine (G) nucleotides into the mRNA of the P protein gene to permit access to additional ORFs (38). The V proteins of the respiroviruses, morbilliviruses, and henipaviruses share the N-terminus as their respective P proteins, which, at the editing site, are joined to a unique, C-terminal cysteine-rich domain encoded by a different ORF (Fig. 3). In contrast, faithful transcription of the rubulavirus P protein gene produces a V protein, while the P protein is produced by RNA editing. The P protein genes of the henipaviruses also code for a C protein, which is produced by internal ribosomal entry from an overlapping reading frame located near the 5' terminus of the P protein gene mRNA. As in the case of the morbilliviruses, the translational start site for the C protein of HeV and NiV is located downstream of the start codon for the P/V protein (19, 30). The P protein genes of HeV and NiV also have the capacity to code for a protein that is analogous to the W protein described for Sendai virus (39). W protein is produced by the addition of two nontemplated G proteins at the RNA editing site. The P protein gene of HeV, but not NiV, also contains another short ORF that codes for a small basic protein (SB) with unknown function. While a similar coding region has been located in the P protein genes of vesicular stomatitis virus and Marburg virus, expression of SB has not been demonstrated for HeV (30).

The P gene products from viruses within the subfamily *Paramyxovirinae* have been demonstrated to inhibit both dsRNA signaling (40–43) and IFN production and signaling (44–46). The ability of viruses to inhibit IFN pathways is considered to be important determinants of virulence and host range. As for other paramyxoviruses, the C, V, and W proteins of NiV and HeV function as virulence factors that interfere with the innate immune system. NiV C, V, and W proteins can rescue growth of interferon (IFN)-sensitive viruses, and cells expressing both V and W of NiV block activation of an IFN-inducible promoter in primate cells (47). The V protein sequesters STAT1 and STAT2 in high-molecular-weight complexes, preventing both nuclear transportation and activation (48, 49), while W protein has been shown to sequester STAT1 in the nucleus (50, 51). The P protein also inhibits the nuclear translocation of STAT1 (50). The V and W proteins also block virus activation of the IFN-ß promoter and of an IFN regulatory factor 3-responsive, IFN-stimulated promoter (50). The ability to

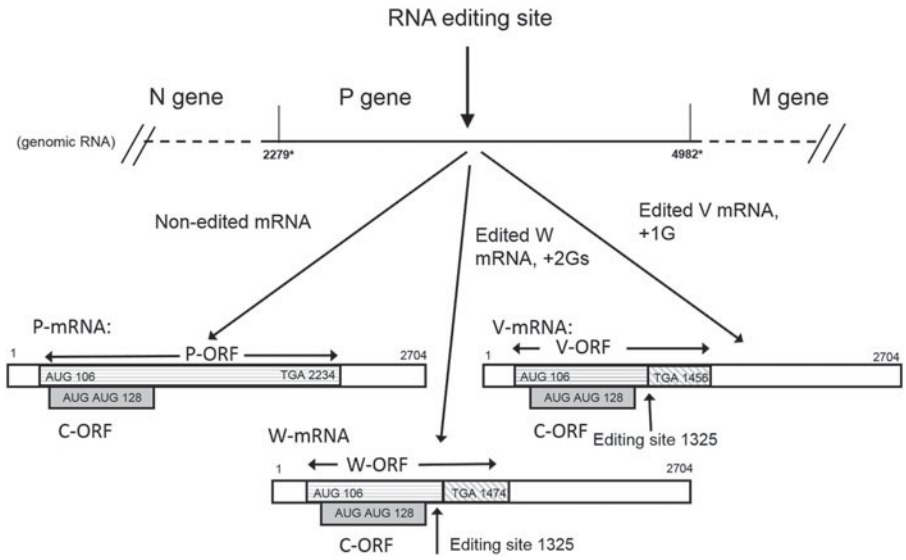

FIGURE 3 Schematic representation of the coding strategy found in the P protein gene of NiV. The predicted P protein mRNA is 2,704 nucleotides in length (nucleotides with asterisks indicate the location of the P protein gene sequence within the sequence with GenBank accession no. AF212302). The P protein is encoded by a faithful transcript of the viral genomic RNA from an opening reading frame beginning at nucleotide 106 of the mRNA. The RNA editing site is indicated by the vertical arrow. The addition of a nontemplated G nucleotide at the RNA editing site (nucleotide 1325) allows access to a different reading frame (–1 relative to P). The V protein contains the amino-terminal domain of the P protein (horizontal lines) joined to the cysteine-rich domain that is unique to the V protein (diagonal lines). The addition of two nontemplated G nucleotides at the RNA editing site produces the mRNA for the W protein in which the amino-terminal domain of P is joined to carboxyl-terminal domain unique for W (diagonal lines). The C protein (gray box) is expressed from an ORF that begins at nucleotide 128 (or 131) and overlaps P in the +1 frame.

block IFN signaling has been mapped to a single amino acid on the NiV V protein (52).

The N, P, and L proteins are necessary and sufficient for transcription and genome replication. The genes coding for the RNA-dependent RNA polymerase (L protein) of HeV and NiV have a linear domain structure that is conserved in all of the *Mononegavirales* (53). In domain III, all of the negative-strand RNA viruses have a predicted catalytic site with the amino acid sequence GDNQ. The sequence QDNE is found only in HeV, NiV, and Tupaia paramyxovirus (3, 31, 54). However, substitution of the E for Q did not affect the function of the L protein of NiV in a minigenome replication assay (55). Interestingly, the GDNE motif is not found in MojPV, Cedar virus (56), and other recently identified bat henipa-like viruses (57).

HeV and NiV have two membrane glycoproteins, the F protein and the attachment protein G, which perform the same functions as the membrane glycoproteins of the morbilliviruses and respiroviruses. As with the other paramyxoviruses, both the G and F proteins of HeV and NiV are required for cell fusion, and heterotypic mixtures of the G and F proteins of HeV and NiV are also fusion competent (58, 59). MenPV lacks detectable neuraminidase and erythrocyte binding activity, and the hemagglutinin-neuraminidases of MenPV and TioPV lack the hexapeptide, NRKSCS, that is proposed to be essential for neuraminidase activity (21). Compared to the other rubulaviruses, TioPV and MenPV also have some unique genetic features in their RNA start sites and intergenic regions (9, 60).

The F proteins of the *Paramyxovirinae* are type I membrane glycoproteins that facilitate the viral entry process by mediating fusion of the virion membrane with the plasma membrane of the host cell. F proteins are synthesized as inactive precursors, F_0, that are converted to biologically active subunits, F_1 and F_2, following proteolytic cleavage by a host cell protease. The F peptide, located at the amino terminus of the F_1 protein, is highly conserved within the *Paramyxovirinae* (61). The F peptides of HeV and NiV are related to the F proteins of other paramyxoviruses, with the exception that HeV and NiV have leucine at the first position, while almost all of the other viruses have phenylalanine (19). However, substitution of phenylalanine for leucine in the F_1 subunit of NiV does not affect its ability to form syncytia (62).

Among the paramyxoviruses, the carboxyl terminus of the F_2 protein subunit contains either single-basic or multiple-basic amino acids that comprise the cleavage site between F_1 and F_2. F proteins with multiple-basic amino acids are cleaved by furin-like protease during exocytosis from the host cell. F proteins of viruses with a single-basic amino acid are cleaved at the cell surface by trypsin-like proteases. These viruses usually require the addition of exogenous trypsin to replicate in cell culture. While HeV and NiV have a single-basic residue at the cleavage site, both produce productive infections in a variety of cell lines in the absence of exogenous trypsin. In addition, the cleavage site of the F proteins of NiV and HeV do not contain a furin-like protease consensus sequence (R-X-R/K-R) found in most morbilliviruses, rubulaviruses, and pneumoviruses (35, 61) and, in fact, the basic amino acids do not appear to be required for cleavage (62). Cleavage of the F protein of NiV and HeV occurs by a novel mechanism involving clathrin-mediated endocytosis via a tyrosine-dependent signal on the cytoplasmic tail (63–65). The F proteins of both HeV and NiV require the endosomal protease cathepsin L for proteolytic processing (66). N-glycans of the F protein of NiV are required for proper proteolytic processing, and these glycans may modulate access to neutralization epitopes (67, 68).

As for other paramyxoviruses, the NiV surface glycoproteins are the primary targets for neutralizing antibodies (59, 69, 70). Recombinant vaccinia viruses expressing NiV F and G proteins elicit neutralizing antibodies against NiV and protect Syrian hamsters and pigs against lethal NiV challenge (59, 70, 71). Antibodies to F or G protein also provided passive protection in the hamster challenge model (70, 72).

Entry and Receptors

The attachment proteins of the *Paramyxoviridae* are type II membrane glycoproteins and are responsible for binding to receptors on host cells (34, 35). Paramyxovirus attachment proteins are designated HN, H, or G, depending on their hemagglutinin (H) and/or neuraminidase (N) activities (73). Unlike many other paramyxoviruses, neither of the henipaviruses has been shown to have erythrocyte binding or neuraminidase activities. The attachment proteins of the henipaviruses are most closely related to the hemagglutinin-neuraminidases of the respiroviruses (74). The conservation of most of the structurally important amino acids suggests that the attachment proteins of HeV and NiV would have structures that are very similar to the structure proposed for the attachment proteins of other paramyxoviruses (61). EphrinB2, the membrane-bound ligand for the EphB class of receptor tyrosine kinases, specifically binds to the attachment (G) glycoproteins of henipaviruses and is a functional receptor for HeV and NiV (75–77). While ephrinB3 has also been shown to be a functional receptor for both viruses, the binding of NiV to ephrinB3 is much more efficient than the binding of HeV (76, 78, 79), and the G protein of NiV has distinct binding regions for ephrinB2 and ephrinB3 (78). EphrinB3 but not ephrinB2 is expressed in the brain stem, so the difference in the abilities of HeV and NiV to bind to these cellular receptors is consistent with the neuroinvasiveness of NiV (76, 78, 79). NiV infection does not appear to down-regulate cell surface expression of ephrinB2 or ephrinB3 (80).

Viral Replication

Cell Culture

Vero cell culture (African Green monkey kidney) supports the growth of HeV, NiV, and MenPV. Both HeV and NiV were first identified as syncytium-forming agents in Vero cell cultures, and electron microscopic studies revealed the presence of typical paramyxovirus "herringbone" nucleocapsid structures (2, 81). Vero cells are routinely utilized to attempt to propagate paramyxoviruses for virus isolation (9, 56, 82–84). In instances where a bat origin is suspected, cell lines such as PaKi (bat kidney) (56, 85, 86) are commonly used for virus isolation from various bat samples.

Virus Stability and Inactivation

Paramyxoviruses are susceptible to common soaps and disinfectants. Lipid solvents (alcohol and ether) and sodium hypochlorite solutions are used effectively in outbreaks for cleaning and disinfection. Paramyxoviruses are stable between pH 4.0 and 10.0 and inactivated by heating at 60°C for 60 minutes.

Animal Models

The development or characterization of animal models to study henipavirus infections has been critical for understanding their pathogenesis and for development of therapeutics or vaccines (87). Both cats and golden hamsters have been used as small animal models, and both develop fatal disease after challenge with NiV. In cats, virus is mostly present in the respiratory epithelium, while hamsters develop neurologic disease (70, 72, 88). NiV in pigs causes a febrile respiratory illness with or without neurologic signs (89, 90). Following infection of fruit bats with NiV, some bats seroconverted and intermittently excreted low levels of virus (91).

EPIDEMIOLOGY

Hendra Virus

Distribution and Geography

There have been multiple outbreaks of HeV among horses recognized between 1994 and 2014. The majority of the outbreaks have occurred in Queensland, Australia, and five have resulted in spillover to humans. The first incident occurred in September 1994 at a stable in Hendra, a suburb of Brisbane. An outbreak of acute respiratory disease resulted in 14 horse deaths (18, 92). Approximately 1 week after exposure to the index horse case, a pregnant mare recently moved to the stable, a stable hand and a horse trainer developed an influenza-like illness. The stable hand recovered completely after 6 weeks, but the horse trainer died on the seventh day of illness. The second incident, in October 1995, involved the death in Brisbane of a farmer from Mackay (93). The patient lived on a horse stud farm and often assisted a veterinary surgeon during treatment. In August 1994, the patient developed meningitis shortly after he cared for, and assisted in, the autopsies of two horses that died, one from acute respiratory distress and the other from rapid-onset neurologic symptoms. Both horses were retrospectively diagnosed with HeV (94). The patient recovered completely and remained symptom free for 13 months before his fatal illness, which is believed to have resulted from reactivation of virus that entered a putative latent phase following the initial disease. In the 20 years since the first two HeV outbreaks, there have been three additional outbreaks involving human cases, with two fatalities.

Transmission

Despite the potential for HeV to infect a wide variety of animals under experimental conditions (95–97), horses appear to be the primary source of human HeV infection. Each of the HeV-infected persons reported extensive contact with horses. In the first incident, both infected persons had close contact with a dying mare, particularly the horse trainer with fatal disease, who had abrasions on his hands and arms and was exposed to nasal discharge while trying to feed the incapacitated horse (98). The infected farmer from Mackay in the second incident cared for sick horses and assisted in their autopsies without using gloves, masks, or protective eyewear (93). No serologic evidence of infection was found in 22 other persons who reported feeding or nursing sick horses or participating in their autopsies or in more than 110 other persons associated with, or living near, the affected stables (98, 99). These data indicate that transmission of infection from horses to humans is inefficient and requires very close contact. The urine and saliva from infected horses are important in disease transmission (95), whereas respiratory spread is less likely. Human-to-human transmission of HeV has not been documented, either among domestic contacts or among health care workers (98, 99).

Reservoir

No epidemiological connection could be found between the first two HeV incidents, which occurred within 1 month of each other at locations 1,100 km apart. No evidence of HeV infection was found among horses, other farm animals, or more than 40 species of wildlife in Queensland (100, 101). However, HeV antibodies were detected in several fruit bat species in Queensland, and the virus isolated from a fruit bat was indistinguishable from that isolated from horses and humans (102–104). The natural reservoir of HeV has been identified as flying foxes or fruit bats, with seroprevalence varying from 10 to 50% in different colonies (103, 105). HeV has also been isolated on several occasions from three different species of Australian pteropid bats: *Pteropus alecto*, *P. poliocephalus*, and *P. conspicillatus* (32, 103). It is postulated that transmission to horses may occur through the ingestion of pasture recently contaminated by the urine or infected fetal tissue of fruit bats (102, 106). In recent years HeV has also been reported to naturally infect dogs. On two separate occasions dogs have tested positive for infection, with these dogs being present on properties with known HeV infected horses. In 2011, a dog tested seropositive to HeV on a property where three horses were infected with HeV, and, in 2013, another dog tested positive to HeV by PCR (107).

Nipah Virus

Distribution and Geography

The first known human infections with NiV occurred during an outbreak of severe encephalitis in 1998 to 1999, during which 265 patients (40% fatal) with viral encephalitis and 11 patients (1 fatal) with laboratory-confirmed NiV disease were reported in peninsular Malaysia and Singapore, respectively (2, 108, 109). This outbreak began in October 1998 in Malaysia near the city of Ipoh and then spread southward in conjunction with the movement of pigs, resulting in three other clusters of human disease in Malaysia. The largest, accounting for approximately 85% of all cases of outbreak-associated encephalitis, occurred in the Bukit Pelandok area of Negeri Sembilan state, a region with extensive pig farming activities. In Singapore, abattoir workers, who slaughtered pigs imported from outbreak-affected areas in Malaysia, were exclusively affected (110, 111). Adult males of Chinese or Indian ethnicity, who were primarily involved in pig farming activities, accounted for more than three-fourths of the cases. Infections were also documented among abattoir workers (110, 111), veterinary personnel and military personnel involved in pig-culling activities to control the outbreak. Since the 1998–1999 outbreak, Malaysia has reported no further cases of NiV infection; however, the virus continues to spill over and cause disease in other countries. Since 2001, human cases of NiV have occurred almost annually in Bangladesh and sporadically in neighboring India (1, 112, 113).

Sequence analyses suggest that there were at least two introductions of NiV into pigs prior to the outbreak in 1999. Only one of these variants was associated with the explosive spread within pig farms and subsequent transmission to humans, suggesting that a single spillover from the reservoir triggered the outbreak. In contrast, the sequence heterogeneity observed between samples obtained from the outbreak in Bangladesh in 2004 suggests multiple spillovers between the reservoir and humans (4).

Transmission

The transmission route of NiV differed between the outbreaks in Bangladesh and India, compared with Malaysia. Human infections in Malaysia were almost exclusively associated with contact with infected pigs (114). In contrast, in Bangladesh, no intermediate animal host has been identified, and human-to-human transmission has been observed (1, 115). Mortality rates of outbreaks in India and Bangladesh have also been higher than the rate reported for Malaysia (116, 117). Direct, close contact with pigs was the primary source of human NiV infection during the initial outbreaks (47, 118). Activities involving close contact with pigs (e.g., medicating sick pigs and assisting in birthing) were associated with the greatest risk of human infection (119). In pigs, extensive infection of the upper and lower airways is seen with evidence of tracheitis and bronchial and interstitial pneumonia, and a harsh, nonproductive cough is a prominent clinical feature (2, 120). Vasculitis of small vessels in the kidney is also seen (2, 120), and viral antigen is detected by IHC studies as focal staining in renal tubular epithelium. Therefore, exposure to respiratory secretions and possibly the urine of infectious pigs likely results in transmission of virus among pigs and to humans.

Drinking fresh date palm sap was the most strongly associated risk factor among the exposures investigated during an outbreak of human NiV infection in Bangladesh in 2008 (121). To prevent NiV transmission, date palm sap should not be drunk fresh unless effective steps have been taken to prevent bat access to the sap during collection.

Serologic studies demonstrated evidence of infection among other species of animals, including dogs and cats (2, 120) on and near farms with NiV-infected pigs. As NiV can be found by IHC staining in renal glomeruli of infected dogs and cats and virus can be isolated from the urine of experimentally infected cats, virus may be transmitted by exposure to the urine of these two species. It is possible that humans are at risk from exposure to infected animals other than pigs, as some patients reported no direct contact with pigs and others reported contact with dogs that died of unknown causes (119, 122).

Although NiV is excreted in respiratory secretions and urine of patients (123), a survey of healthcare workers during the first outbreak initially demonstrated no evidence of human-to-human transmission (124). Human-to-human transmission has since been confirmed (125–127) and is a major pathway for human NiV infection (128).

Reservoir

Knowing the similarities between NiV and HeV, surveillance for the natural reservoir for NiV focused on bats. Neutralizing antibodies to NiV were found in a total of 21 bats belonging to four fruit bat species and one insectivorous bat species in peninsular Malaysia (104). Attempts to detect the virus in sera from bats using both cell culture and amplification of RNA were unsuccessful. Bat colonies were noted proximal to pig farms near the city of Ipoh, where the outbreak in pigs was first noticed, supporting the hypothesis that transmission from bats to pigs initiated the outbreak (104).

Evidence of henipaviruses in several species of the genus *Pteropus* has suggested that the geographic range of the henipaviruses is significant and ranges from African coastal islands to the Western Pacific Islands (Fig. 4), including American Samoa, Thailand, Cambodia, Indonesia, Bangladesh, and Madagascar (112, 129–135). These findings primarily implicate species within the genus *Pteropus*, suggesting that this genus shares a coevolutionary relationship with the genus *Henipavirus*.

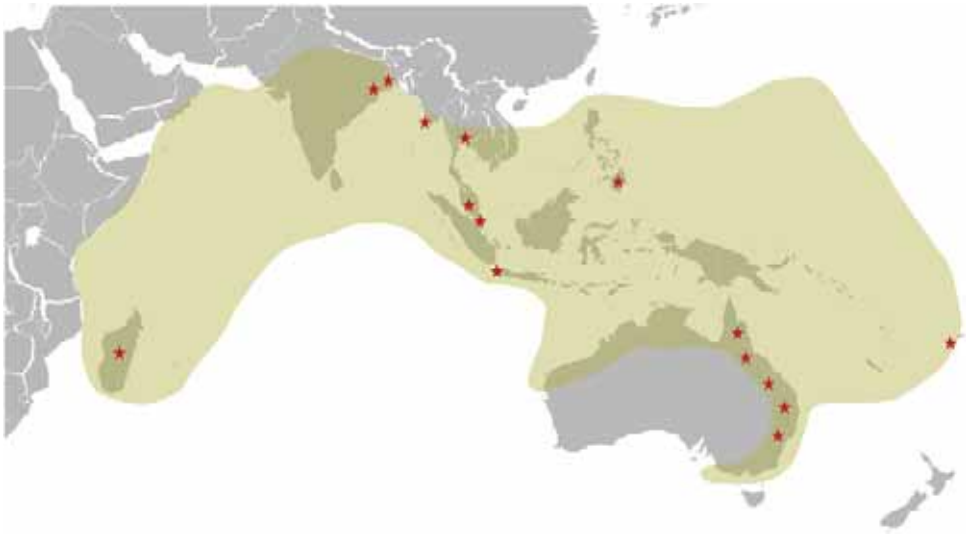

FIGURE 4 Range of the genus Pteropus (shaded area). Locations where human infections of henipaviruses have occurred are designated by stars.

Menangle Virus

Distribution and Geography

From mid-April to early September 1997, at a piggery in New South Wales, Australia, a decline was noticed in the farrowing rate of sows, associated with an increase in the proportion of malformed, mummified, and stillborn piglets and occasional abortions (6, 7). Affected piglets had craniofacial and spinal abnormalities and degeneration of the brain and spinal cord. A new paramyxovirus, MenPV, was isolated from the brain, heart, and lung specimens of several affected piglets. No disease was seen in postnatal pigs of any age, but a high proportion of serum specimens (>95%) collected from these animals contained high titers of antibodies that neutralized the virus. Evidence of infection with MenPV was also detected in porcine sera from two other associated piggeries that received weaned pigs from the affected piggery but not in sera from several other piggeries throughout Australia (136).

A serologic survey of persons who came into contact with the affected piglets (5) detected a high titer of neutralizing antibodies in two workers, one at the affected piggery and one at an associated piggery. Both workers had an influenza-like illness concomitant with the outbreak in pigs, and no alternative cause was identified despite serologic testing. Thus, the illness was attributed to MenPV infection.

Transmission

Close contact with infected piglets appears to be the primary mode of transmission of MenPV to humans. The worker reported splashes of amniotic fluid and blood to the face and the frequent occurrence of minor wounds on his hands and forearms (5). The other worker performed autopsies on pigs without gloves or protective eyewear. Of note, a large breeding colony of fruit bats roosted within 200 meters of the affected piggery, and sera from several bats had antibodies that neutralized MenPV (6). In addition, antibodies were found in sera collected in 1996 before the outbreak and from a colony of fruit bats 24 kilometers from the piggery. All other sera collected from a variety of wild and domestic animals (e.g., cattle, sheep, birds, rodents, feral cats, and a dog) in the vicinity of the affected piggery tested seronegative for the virus.

In 2009, MenPV was isolated from a bat roost at Cedar Grove, South East Queensland, Australia (86). Black flying foxes were the predominant species in this colony at the time of sampling. The virus isolated was sequenced and demonstrated a 94% nucleotide sequence identity to the virus isolated from pigs in 1997, providing strong evidence supporting the original hypothesis that the outbreak of MenPV infection in pigs and humans in 1997 was probably the result of a spillover from bats roosting near the piggery.

Sosuga Virus

Distribution and Geography

In 2012, a wildlife biologist returned to the United States and developed a severe acute febrile illness after spending 6 weeks in South Sudan and Uganda collecting bats and rodents from remote rural areas for ecological research (8). During the field trip, the biologist manipulated animals in traps and mist nets, performed dissections, collected blood and tissues, and visited caves with large bat populations. Initial diagnostic evaluation excluded malaria, common bacterial pathogens, and viral hemorrhagic fever viruses like Ebola and Marburg. Next generation sequencing was performed on RNA extracted from blood and serum samples of the patient, and metagenomic analysis revealed a novel paramyxovirus (8) most closely related to ThkPV-3, a rubula-like virus isolated from *Rousettus leschenaultii* fruit bats in southern China (36). The novel paramyxovirus was provisionally named Sosuga virus (SosPV) in recognition of its probable geographic origin (South Sudan, Uganda). To date, there has only been a single recorded case of SosPV infection.

Transmission

It is unclear how exactly the biologist became infected with SosPV. Interviews with the patient revealed that personal protective equipment (PPE) was used during animal capture and processing in Kibaale, Uganda, but inconsistent use of PPE occurred during the earlier South Sudan work (137).

Sequence similarity of SosPV with other bat-derived rubula-like viruses, in addition to the circumstances surrounding the biologist's illness is highly suggestive of a bat origin for this new paramyxovirus (8).

SosPV was detected in Egyptian fruit bats (*Rousettus aegyptiacus*), whereas Ethiopian epauletted fruit bats (*Epomophorus labiatus*), Angolan rousettes (*Lissonycteris angolensis*), and a roundleaf bat (*Hipposideros spp.*) caught in the same general vicinity were negative (137). Egyptian rousette populations in multiple locations across Uganda were shown to be actively infected with SosPV over a 3-year period (137). Given the biologist's exposure to bats in Uganda during the 3 weeks prior to onset of illness, these Egyptian rousettes were the probable source of the infection. The wide distribution and detection of the virus at multiple time points suggest the Egyptian rousette could be a reservoir species (137).

Suspected Zoonotic Paramyxovirus Infections

Many novel paramyxoviruses have been detected across the world, including Ghana, Zambia, Democratic Republic of Congo, Gabon, Central African Republic, Comoros, Mauritius, Madagascar, Germany, Bulgaria, Romania, Thailand, Indonesia, Costa Rica, Panama, Brazil, and the Philippines (57, 83, 84, 138–143). These viruses belong to the genera *Henipavirus*, *Morbillivirus*, *Rubulavirus*, and *Pneumovirus*. Several of these novel paramyxoviruses are either implicated or suspected in causing zoonotic diseases.

Tioman virus (TioPV) was isolated from urine of the island flying fox (*Pteropus hypomelanus*) collected from Tioman Island off the eastern coast of peninsular Malaysia (9). TioPV is closely related to MenPV and has also been tentatively classified in the genus Rubulavirus. A serological survey of 169 Tioman Islanders demonstrated five individuals (1.8%) had neutralizing antibodies to TioPV, suggesting previous infection with TioPV or a similar virus (11). Successful experimental infection of pigs suggested that pigs could become naturally infected with TioPV and could facilitate virus transmission to humans following contact with oral secretions (10). However, there is currently no evidence of TioPV causing disease in humans or animals.

Mojiang virus (MojPV) is a novel henipa-like virus detected in rats (*Rattus flavipectus*) in Yunnan Province, China, in 2012 (17). This virus was detected in rectal swabs collected from rats in a cave, cohabitated by bats (*Rhinolophus ferrumequinum*). The detection resulted from part of an investigation of three fatal human cases among workers who had been involved in cleaning the cave, although no causative relationship can be established. Six months earlier, three humans visiting this cave had developed severe pneumonia. Although isolated from rats, it is not clear whether rats are the natural reservoir or a spillover host in this case. Genome sequence analysis of MojPV demonstrated that the closest relatives are the henipaviruses. If rats are the natural reservoir of this virus, it would suggest that henipaviruses have a broader natural reservoir range than just bats.

Achimota viruses (AchPV1 and 2) are two different paramyxoviruses, which have been isolated from urine samples collected from straw-colored fruit bat (*Eidolon helvum*), bats roosting in Accra, Ghana (16). These two viruses, although not closely related, cluster with the other bat-borne rubula-like viruses. Human sera collected in Ghana and Tanzania from both healthy and febrile humans were able to neutralize AchPV2 (16). This suggests that AchPV2 has zoonotic potential, with either AchPV2 or a closely-related virus having infected humans in the past. Whether these viruses can cause disease in humans or animals is currently unknown.

During 2014, an outbreak of severe illness among humans and horses was reported in the villages Tinalon and Midtungok, province of Sultan Kudarat, island of Mindanao, Philippines (143). The outbreak encompassed fatal and nonfatal human infections, as well as concurrent neurologic disease (head tilting, circling, ataxia) and sudden deaths in several horses, which were subsequently consumed by villagers. A total of 17 human cases were identified during this outbreak. Of these cases, 7 people had participated in horse slaughtering and horse meat consumption, and 3 had only consumed horse meat. A further 5 people had been exposed to other infected humans, and, of these, 2 were healthcare workers without other known contacts (143). Among other domestic animals, 4 cats and 1 dog that had eaten horse meat died. Virus isolation was unsuccessful, but neutralizing antibodies and IgM against NiV and correspondingly lower neutralizing antibody titers against HeV were found for 3 patients (143). The pattern of neutralizing antibodies and IgM in paired serum samples was interpreted as evidence of recent exposure to a henipavirus. In addition, a serum sample from 1 of these patients was positive by real-time PCR for NiV, and a single-sequence read (71 bp) of the P gene of NiV was detected by next generation sequencing of the CSF from another of these patients. In addition to transmission to humans by direct exposure to infected horses, their contaminated body fluids or consumption of undercooked meat from infected horses, there is strong evidence for direct human-to-human virus transmission. Although the source of the horse infections is unclear, on the basis of the known ecology of henipaviruses, the most likely source of horse infection is fruit bats (143).

PATHOGENESIS

Hendra Virus

A total of seven human HeV infections have occurred to date, with four patients succumbing to their infections. All human infections have resulted from close physical contact with infected horses. Symptoms have varied between patients, with an estimated incubation period of 7 to 16 days. Initial disease signs are influenza-like, which progress to a fulminating encephalitis with multiorgan, failure (98, 144). The autopsy of the horse trainer who died in the first HeV incident showed a severe interstitial pneumonia. Both lungs were congested, hemorrhagic, and edematous. Histologic examination showed focal necrotizing alveolitis, with giant cells, syncytium formation, and viral inclusions. Postmortem lung, liver, kidney, and spleen samples were inoculated in cell culture, resulting in the appearance of prominent syncytia in cultures inoculated with kidney material (18, 98). The findings at autopsy of the farmer from Mackay, who died more than 1 year after initial infection with HeV (93), showed leptomeningitis with lymphocyte and prominent plasma cell infiltration, with discrete foci of necrosis in the neocortex, basal ganglia, brain stem, and cerebellum. Multinucleate endothelial cells were observed in the brain, liver, spleen, and lungs. Immunohistochemical (IHC) studies showed the presence of viral antigen in the cytoplasm of some cells, but most frequently scattered throughout the neurophils, although virus could not be isolated from the brain. These findings suggest that, following initial infection with the virus, possibly through the oral/respiratory route or through direct inoculation of cutaneous abrasions with

infectious secretions, viremia develops, resulting in spread to various organs, including the central nervous system. The pathogenesis of recurrence of fatal disease in the farmer from Mackay is unclear. The antibody profile during the fatal illness was indicative of an anamnestic response to viral antigens (93). This patient had abundant immunoglobulin M (IgM) antibodies suggestive of a reinfection, but no exposure to horses was documented prior to the recurrence. The failure to isolate virus from this patient's brain supports speculation that the pathogenesis may be similar to that of subacute sclerosing panencephalitis, as both diseases are caused by paramyxoviruses and are characterized by recurrence of fatal neurologic disease following complete recovery from an initial infection. However, the pathological findings and rapid course of this disease are strikingly different from those of subacute sclerosing panencephalitis.

Nipah Virus

The incubation period for NiV infection has been estimated to be 1 to 2 weeks. During the initial outbreak, the period between the last contact with pigs and onset of illness ranged from several days to 2 months, but it was 2 weeks or less for 92% of patients (119). All available data on histopathological changes in humans infected with Nipah virus were obtained in a single study performed during the Nipah virus outbreak in Malaysia and Singapore (145). A multiorgan vasculitis associated with infection of endothelial cells is the hallmark pathological feature of Nipah disease (122). Occasionally, multinucleate giant cells characteristic of paramyxovirus infections are observed in the affected vascular endothelium. Infection is most pronounced in the central nervous system, where a diffuse vasculitis, characterized by segmental endothelial cell damage, mural necrosis, karyorrhexis, and infiltration with polymorphonuclear leukocytes and mononuclear cells, is noted. The lesions are primarily seen in the cerebral cortex and brain stem, with extension to parenchymal tissue, where extensive areas of rarefaction necrosis are seen. Eosinophilic, mainly intracytoplasmic, viral inclusions with a "melted-tallow" appearance are seen in the affected neurons and parenchymal cells. IHC studies with NiV antigen show intense staining of endothelial and parenchymal cells. Evidence of endothelial infection and vasculitis is also seen in other organs, including the lungs, heart, spleen, and kidneys. NiV has been isolated from CSF, tracheal secretions, throat and nasal swabs, and urine specimens from patients (119, 122).

The widespread distribution of vasculitis throughout the central nervous system and, to a lesser extent, in other organs, and the isolation of virus from a variety of clinical specimens, suggest that, following initial infection with the virus, possibly through the respiratory tract or direct inoculation of cutaneous abrasions with infectious secretions, viremia develops, resulting in systemic spread. Involvement of the uncus of the temporal lobe in some patients has led to speculation that virus may be spread along the olfactory tract to the uncus following inhalation and local replication (146). The nonspecific neurologic manifestations of Nipah disease probably reflect widespread vasculitis, but the distinctive features, such as segmental myoclonus and brain stem dysfunction, indicate a predilection of the virus for certain neurons.

Findings at autopsy of a patient, who died of encephalitis that developed 10 weeks following an initial asymptomatic infection, showed evidence of neuronal death, neuronophagia, parenchymal inflammation, and perivascular cuffing, suggestive of a primary viral encephalitis rather than a vasculitis or infarction (146). MRI showed patchy areas of confluent cortical involvement, mainly in the cerebral hemispheres. The distinctive pathology and the MRI features of patients with relapse suggest that the pathogenesis may differ from that of acute Nipah encephalitis.

Immune Responses

Limited data are available regarding the human immune response to NiV infection and correlates of immune protection and disease resolution. A serum IgM response has been demonstrated shortly after onset of illness, and the presence of IgM antibody appears to reduce the rate of isolation of virus from throat and respiratory secretions (123). The presence of antibodies in the serum or CSF, however, does not appear to influence the rate of isolation of virus from CSF (81), suggesting that humoral immunity plays a minor role in recovery from neurologic disease.

The NiV nonstructural proteins C, V, and W have been shown to play a role in pathogenesis by antagonizing the interferon signaling response (147). Specifically, the C protein inhibits the early proinflammatory response at sites of infection, thereby preventing control of the infection by the immune system (148). NiV C regulates expression of proinflammatory cytokines, therefore providing a signal responsible for the coordination of leukocyte recruitment and the chemokine-induced immune response and controlling the lethal outcome of the infection. The role of innate immune signaling in NiV pathogenesis is further exemplified by the fact that NiV causes disease in IFNAR-KO mice lacking the type I interferon receptor, which are thus deficient in interferon signaling, but not in wild-type mice (149).

CLINICAL MANIFESTATIONS

Hendra Virus

The two patients in the first HeV incident had abrupt onset of an influenza-like illness, characterized by myalgia, headaches, lethargy, and vertigo (18, 92). One patient remained lethargic for 6 weeks but fully recovered. The other patient developed nausea and vomiting on the fourth day of illness and deteriorated rapidly in the next 2 days, requiring admission to an intensive care unit and mechanical ventilation. He died on the seventh day of illness. The fatal patient in the first HeV incident showed thrombocytopenia; increased levels of creatine phosphokinase, lactic dehydrogenase, aspartate aminotransferase, alanine aminotransferase and glutamyltransferase; and features of dehydration and acidosis (18). Chest radiographs showed diffuse alveolar shadowing. No laboratory abnormalities were detected in the patient who survived.

Unlike the first two patients, the affected farmer in the second HeV incident primarily had neurologic manifestations (93). He initially presented with features of meningitis, including headache, drowsiness, vomiting, and neck stiffness. Thirteen months following complete recovery, the patient presented again with a 2-week history of irritable mood and lower back pain, three episodes of focal seizures of the right arm, and an episode of generalized tonic-clonic seizures. In the following week, he continued to have a low-grade fever and focal and generalized seizures. By day 7, he developed dense right hemiplegia, signs of brain stem involvement, and depressed consciousness, requiring intubation. The patient remained unconscious and febrile until he died, 25 days after admission. Cerebrospinal fluid (CSF) examination showed an elevated protein level, normal glucose level, and mononuclear pleocytosis. Magnetic resonance imaging

(MRI) of the brain showed multifocal cortical lesions, sparing the subcortical white matter, that became more pronounced and widespread prior to death. The two patients infected during the 1998 outbreak also experienced initial influenza-like symptoms; however, after apparent clinical improvement, encephalitis developed in both patients. The MRI changes showed widespread cortical, subcortical and deep white matter involvement, similar to those described in a previous HeV encephalitis case (144).

Nipah Virus

The initial symptoms of NiV are nonspecific and include headaches, fever, dizziness, and muscle pain. As the disease progresses, neurological symptoms become the dominant feature and, depending on the strain, respiratory involvement to various degrees. The onset of NiV disease is abrupt, usually with the development of fever. Often, patients deteriorate rapidly, requiring hospitalization 3 to 4 days after onset of symptoms. Severe encephalitis is the most prominent clinical manifestation. Fever (97%), headache (65%), dizziness (36%), vomiting (27%), and reduced level of consciousness (21%) are the most common features at presentation (122). Several other features of neurologic involvement, particularly signs of brain stem dysfunction, are noted in patients during the course of illness (Table 2). The disease in 3 of the 11 patients in the initial Singapore outbreak presented as an atypical pneumonia, with fever and infiltrates on chest radiography (111). NiV disease was fatal in up to one-third of hospitalized patients in Malaysia. The following are all associated with a poor prognosis: older age; evidence of brain stem involvement; the presence of segmental myoclonus, seizures, or areflexia; elevated hepatic enzyme levels or low platelet counts; and isolation of virus from the CSF (81, 122).

Survivors of NiV infection frequently experience long-term neurological deficits (150). Late-onset or relapse encephalitis has been observed, and, in one case, this late-onset encephalitis occurred 11 years after the initial NiV infection (150, 151).

Laboratory Abnormalities

Thrombocytopenia (30%), leukopenia (11%), and elevated levels of alanine aminotransferase (33%) and aspartate transaminase (42%) are the most common hematologic abnormalities (122). CSF studies are frequently abnormal, with elevated white blood cell counts and/or protein levels (111, 122), but the presence of abnormal CSF findings does not correlate with severity of disease (81).

Computed tomography scans of the brain are generally unremarkable. On MRI, small, discrete lesions measuring 2 to 7 millimeters are seen in the subcortical and deep white matter of the cerebral hemispheres during both the acute and late phases of illness (111, 122, 146). These lesions possibly represent focal areas of ischemia and infarction resulting from the vasculitis. The pattern and extent of brain involvement on MRI do not appear to correlate with specific clinical features, severity of coma or outcome of disease (118). Electroencephalography (EEG) shows the following abnormalities: diffuse slow waves with focal sharp waves; continuous, diffuse, irregular slow waves; and intermittent, diffuse, slow waves (122, 146). Focal EEG abnormalities occur primarily in the temporal lobes.

TABLE 2 Neurologic features in patients with laboratory-confirmed NiV disease, Malaysia, 1998 to 1999[a]

Feature	% of patients (n = 94)
Absent or reduced reflexes	56
Abnormal pupils	52
Tachycardia (heart rate >120/min)	39
Hypertension (blood pressure >160/90 mm Hg)	38
Abnormal doll's-eye reflex	38
Segmental myoclonus	32
Meningism	28
Seizures	23
Nystagmus	16
Cerebellar signs	9
Bilateral ptosis	4

[a]Adapted from reference (113) with permission of the Massachusetts Medical Society.

Complications

Residual neurologic deficits, including a vegetative state, cognitive impairments, and cerebellar disabilities, occur in 10 to 15% of patients (111, 122, 146). Recurrence of neurologic dysfunction is seen in some patients, including neurologic relapse with seizures and cognitive impairment or focal signs, such as isolated cranial nerve dysfunction.

Even without delayed progression to neurologic illness following Nipah fever, persistent fatigue and functional impairment are frequent (150). Neurologic sequelae were frequent following Nipah encephalitis. Neurologic dysfunction may persist for years after acute infection, and new neurologic dysfunction may develop after acute illness. Survivors of NiV infection may experience substantial long-term neurologic and functional morbidity.

Clinical Diagnosis

Encephalitis can be diagnosed by the presence of fever, headache, reduced level of consciousness, and focal neurologic signs, as well as abnormalities on CSF examination and EEG studies. Clues to the NiV etiology are provided by the history of contact with pigs or bats, particularly in the context of an outbreak, the presence of segmental myoclonus and MRI findings of small, discrete lesions in the subcortical and deep white matter of the cerebral hemispheres. MRI findings are particularly useful in distinguishing encephalitis caused by NiV from that caused by Japanese encephalitis virus, the most common arboviral encephalitis worldwide, which is endemic in China, India, and other parts of Southeast Asia, and by herpes simplex virus, the most common sporadic form of encephalitis worldwide.

Menangle Virus

The two MenPV-infected workers had similar illnesses, characterized by abrupt onset of fever, malaise, chills, drenching sweats, and severe headache (5). On the fourth day of illness, both developed a spotty, red, nonpruritic rash. Bilateral hypochondrial tenderness was present in one patient, and an abdominal ultrasound conducted 2 months after the illness showed splenomegaly and liver size at the upper limit of normal. Both patients recovered after approximately 10 days of illness.

Sosuga Virus

Symptoms upon hospital admission included a 2-day history of fever, malaise, headache, generalized myalgia, and

arthralgia, neck stiffness, a metallic taste, and sore throat (8). A maculopapular rash on the trunk erupted on the second day after hospital admission, and several small ulcers appeared on her soft palate. The following day, fever, headache and myalgia persisted, and the patient experienced bloody emesis and mild diarrhea positive for occult blood but without frank hematochezia or melena. The rash became confluent. A bone marrow biopsy sample showed a mild increase in macrocytic hemophagocytosis and pancytopenia with a hypocellular marrow with myeloid hyperplasia and erythroid hypoplasia. The fever slowly but progressively decreased, and the last recorded fever was on the ninth day in hospital. The biologist was discharged from hospital after 2 weeks, but considerable sequelae (myalgia, arthralgia, headache, malaise, and fatigue) persisted for several months (8).

LABORATORY DIAGNOSIS

Virus Isolation

Traditional techniques of virus isolation in cell culture, electron microscopy, enzyme-linked immunosorbent assay-based serology, neutralization assays, and IHC techniques have been employed in the diagnosis of the zoonotic paramyxoviruses (2, 6, 152, 153). Virus replication, hence success of isolation, is cell-line dependent. NiV has been successfully isolated from human specimens, including nasal and throat swabs, as well as urine and CSF (122). The kidneys have been the only human source of HeV isolation from humans (18). In contrast, MenPV has not been isolated from human specimens, but virus isolations from the tissues of stillborn piglets were readily obtained (6).

NiV and HeV are internationally classified as biosafety level or biosecurity level 4 (BSL-4) agents; thus, clinical specimens suspected to be infected with these agents must be handled with caution. Propagation of viruses from clinical specimens known to be infected with henipaviruses is not recommended without appropriate containment facilities. The US Centers for Disease Control and Prevention, Atlanta, GA, and the Australian Animal Health Laboratory, Geelong, Australia, have adopted the approach that primary virus isolation from specimens of outbreaks, not already proven to be henipaviruses, take place at BSL-3 facilities. However, if the results of cell culture suggest the presence of these agents, cultures should be transferred to BSL-4 facilities to conform to biosafety guidelines (152).

Antigen and RNA Detection

IHC is an excellent technique for the detection of the zoonotic paramyxoviruses (2, 94, 152, 153) in the absence of BSL-4 facilities. Tissue specimens can be formalin-fixed with minimal risk to the laboratory workers. Convalescent-phase human serum was initially used for antigen detection in the investigations of the first HeV outbreak, but subsequently, a wide variety of immunologic reagents became available from the Australian Animal Health Laboratory and US CDC, including polyvalent and monoclonal antibodies to both HeV and NiV. HeV and NiV are genetically closely related (2, 19); thus, the investigation of more current NiV outbreaks benefited greatly from the availability of immunologic reagents made to HeV.

Diagnostic specimens from suspected cases can be disrupted in chaotropic salts in preparation for RNA extraction and RT-PCR. The addition of the chaotropic agent (guanidinium isothiocyanate) almost immediately abrogates paramyxovirus infectivity, and it minimizes human exposure to the infected tissues. RT-PCRs with conserved primer pairs (or families of degenerative primers), flanking the P protein gene-editing region (2, 38) of the subfamily *Paramyxovirinae*, were utilized in the investigation of the NiV outbreak in Malaysia and Singapore. Consensus primers targeting the conserved region of the L gene (154) have been successfully used in the identification and characterization of AchPV 1 and 2 (16), several henipaviruses in African bats (138, 155), and unclassified paramyxoviruses in Indonesia (141), Europe (57, 83), Africa (57, 139), and South America (57). This method can be used in conjunction with fresh or formalin-fixed tissues from a variety of sources, including brain, lung, and kidney, as well as CSF, for the detection of viral sequences.

Next-generation sequencing (NGS), otherwise known as massively parallel or deep sequencing, (156, 157) functions without knowledge about the target sequence, as opposed to conventional PCR. Targeted enrichment can greatly enhance the discovery rate of novel viruses (158–162). A combination of NGS and metagenomic analysis was used for the identification of SosPV (8), AchPV (16), and several other paramyxoviruses (163, 164).

Serology

Enzyme-linked immunosorbent assays, using both indirect and antibody capture formats, have been configured for the detection of IgM and IgG antibodies to HeV and NiV (152). However, viral antigen preparation for the henipaviruses is expensive and must take place in a BSL-4 facility. While sufficient quantities were prepared and made available for the diagnostic needs of the HeV and NiV outbreaks, alternative approaches to viral antigen production from virus-infected cells are currently being explored. One alternative is the expression of individual viral proteins following the incorporation of the viral genes in baculovirus expression or similar expression systems. Measuring serum neutralization antibody titers against HeV and NiV is currently performed in Vero cell monolayers at BSL-4 facilities. In order to negate the requirement for BSL-4 containment, which is not widely available, pseudotyped virus particles provide an alternative diagnostic method that can be performed at BSL-2 conditions. VSV pseudotype particles displaying NiV F and G were used as a substitute for NiV virions (165, 166). Multiplexed microsphere assays for henipaviruses have also been used as a surrogate for virus neutralization (167). These tests are very specific and have been used in conjunction with enzyme immunoassays and radioimmunoprecipitation assays to confirm acute infection and previous exposure to the viruses. In addition, the neutralization assay has been used to detect neutralizing antibody to HeV and NiV in animal serum specimens, particularly bat serum, in attempts to identify possible reservoirs of these agents (56, 104, 105, 152, 168).

PREVENTION

Currently, the only vaccine that exists for a bat paramyxovirus is Equivac HeV (Zoetis, Parkville, VIC., Australia), the HeV vaccine approved for use in horses. Equivac HeV was launched in 2012 and is the first vaccine licensed and commercially deployed against a BSL-4 agent (97). The HeV subunit vaccine consists of a recombinant soluble and oligomeric form of the G glycoprotein (169). Vaccine efficacy in immunized horses was assessed against the clinical, virologic, and pathologic features of HeV infection (97, 170). Studies using NiV in cats (88, 171) and monkeys (172) and HeV in ferrets (173) and nonhuman primates (174) provided strong evidence that the HeV glycoprotein subunit-based

vaccine could prevent not only disease but also infection in animals exposed to otherwise lethal doses of either HeV or NiV. The HeV vaccine has the potential for breaking the chain of HeV transmission from bats to horses to humans, thereby protecting both horse and human health.

Apart from vaccination, the principal means of preventing human infections are early recognition of animal disease and use of precautions to avoid exposure. As interruption of transmission to horses or pigs from the natural reservoir of these viruses is difficult, early identification of infected animals and use of appropriate personal protective measures to prevent transmission are keys to reducing the risk to humans. For example, persons handling horses in areas in which HeV may be endemic should be educated regarding the features of disease in horses and should use appropriate PPE (e.g., gloves, gowns, and face visors).

Pig farmers in areas in which NiV may be endemic should be educated regarding the features of Nipah encephalitis in pigs and to report any unusual disease. As transmission is possible without close contact with pigs, exposure to potentially infected animals should be completely avoided, if possible. Persons handling pigs or their excreta should wear PPE such as gloves, masks, gowns, and face visors.

Infection in piggeries with NiV or MenPV can be eradicated through a combination of quarantine, segregation, and culling. During the outbreak in Malaysia, a national swine testing and surveillance program was initiated in which a sample of adult sows from pig farms was tested for antibody to NiV. Farms with antibody-positive animals were considered infected, and pigs from these farms were culled. A similar program to identify infected farms by testing pigs entering abattoirs is also important.

During community outbreaks, additional control measures may be required, including the restriction of movement of animals between farms, the culling of pigs from infected farms and the temporary closure of abattoirs that slaughter pigs from farms in outbreak-affected areas. Disinfection of fruit and boiling of palm sap potentially contaminated by bats are warranted in areas where repeated NiV outbreaks have occurred (115, 175). In areas where large populations of fruit bats of the genus *Pteropus* congregate or frequent fruit or other agricultural products, means of reducing exposure of persons to potentially contaminated plants, areas and products and means of disinfection of fruits or commodities before consumption should be considered.

Person-to-person transmission of NiV occurred in Bangladesh and perhaps in India (1, 115, 126). Given the potential severity of illness, those in contact with patients, including healthcare workers, should use standard and droplet precautions (e.g., strict attention to good hand-washing practices and wearing a mask and gloves) during contact with secretions, excretions, and body fluids of patients.

TREATMENT

Symptomatic therapy includes the use of antipyretic and antiepileptic drugs and mechanical ventilatory support for severely ill patients. Because of the histopathological features of vasculitis-induced thrombosis, aspirin and pentoxifylline were used empirically in some NiV patients (122).

No specific antiviral therapy for infection by henipaviruses has been proven effective to date. One HeV patient recovered completely without any specific treatment, and neither patient with fatal disease received antiviral therapy. *In vitro* ribavirin is inhibitory for HeV at a concentration of 10 to 50 micrograms per milliliter, and the drug also crosses the blood-brain barrier with a mean CSF/plasma ratio of 0.7 (110, 176). In Malaysia, ribavirin was administered either orally or intravenously to 140 patients with suspected Nipah encephalitis; 54 patients who were managed prior to the availability of ribavirin or refused treatment were selected as controls (110). Of the 140 patients who received ribavirin, 128 received it orally (2 g on day 1, 1.2 g three times daily on days 2 to 4, 1.2 g twice daily on days 5 and 6, and 0.6 g twice daily for another 1 to 4 days), whereas 12 received ribavirin intravenously (loading dose of 30 mg/kg of body weight, followed by 16 mg/kg every 6 h for 4 days and 8 mg/kg every 8 h for 3 days). A total of 45 (32%) of the 140 treated patients died, compared with 29 (54%) of the 54 controls, representing an apparent 40% decline in the mortality rate in the treated group compared to historical and untreated controls. Because a small number of patients received intravenous ribavirin, its effectiveness could not be adequately compared with that of oral ribavirin. Despite the absence of any known published information on the *in vitro* effect of acyclovir on NiV infection or replication, acyclovir was empirically administered to all nine patients with encephalitis in Singapore. While only one of these patients died, several deteriorated before recovering. This may have been related to the quality of supportive care provided. The influence of acyclovir therapy on the course of disease is unknown.

Passive immunotherapy, with either polyclonal or monoclonal antibody specific for henipavirus envelope glycoproteins, has proved successful from initial proof-of-concept findings in animal models (70, 72, 177). Presently, the most promising postexposure therapy against Hendra or Nipah virus infection is a human monoclonal antibody (mAb) known as m102.4, which was isolated from a recombinant naïve human phage-displayed Fab library (178, 179). The m102.4 mAb has exceptionally potent neutralizing activity against both Nipah and Hendra viruses, and testing of m102.4 has confirmed its neutralization activity against different isolates of NiV-MY and NiV-BD (177). Effective postexposure efficacy with m102.4 has now been demonstrated in nonhuman primates (180). The cell line expressing the human m102.4 mAb was provided to the Queensland Government, Queensland Health, to allow health authorities to manufacture m102.4 for its potential use on a compassionate basis in future cases of high-risk human exposure. A phase I clinical trial will be conducted in Queensland.

Galectin-1, an endogenous lectin secreted by a variety of cell types, has pleiotropic immunomodulatory functions and also appears to have antiviral effects against NiV (181), although recently this lectin has been shown to also promote NiV infection of endothelial cells (182). A peptide based on the heptad repeats of the hPIV3 F protein was also able to inhibit HeV infection (183). A novel minigenome assay based on polymerase 1-driven transcription has been used to screen a library of small molecules to identify potential lead compounds for further study (184).

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Rhabdoviruses

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Rabies is an acute encephalomyelitis of humans and animals caused by infection with rabies virus. Rabies virus is usually transmitted by an animal bite. Worldwide, dogs are the most important rabies vector, whereas in North America wild animals, especially bats, are the main threat to humans. After a delay at the site of entry, rabies virus spreads through the nervous system within axons by fast axonal transport. Rabies can be very effectively prevented after a recognized animal exposure with wound cleansing and administration of rabies vaccine and rabies immune globulin. Rabies typically develops after an incubation period of 20 to 90 days following the exposure. There are both encephalitic and paralytic forms of rabies and the disease is virtually always fatal after clinical onset. Hydrophobia is a characteristic clinical feature of encephalitic rabies. Progressive weakness involving the limbs and face occurs in paralytic rabies, which often begins close to the site of the wound. Pathological changes in rabies include the presence of eosinophilic inclusions called Negri bodies in the cytoplasm of neurons and inflammatory changes. Imaging studies may be normal and do not show specific abnormalities. A laboratory diagnosis can be made antemortem with the detection of rabies virus antigen or RNA in tissues (e.g., skin) and/or body fluids (e.g., saliva) and with serological testing. There have been rare survivors of rabies, but there is no known effective therapy.

Rhaboviruses in the family *Rhabdoviridae* contain many different virus species, but the most important is rabies virus in the *Lyssavirus* genus that causes the vast majority of cases of rabies. Rabies is an acute viral infection of the central nervous system (CNS) that is almost invariably fatal. Rabies is a zoonotic disease affecting mammals and is normally transmitted to humans by bites from infected animal vectors. Nonrabies virus lyssaviruses have also been recognized to rarely cause disease in humans and animals with identical clinical and pathological features to rabies. Rabies has typical clinical features, but they may not be recognized by physicians who are not familiar with the disease. Rabies can be very effectively prevented after recognized exposures if current recommendations are followed very closely. Vesicular stomatitis virus (VSV) is another rhabdovirus in the *Vesiculovirus* genus that causes vesiculation and ulceration in cattle, horses, and other animals, and causes a self-limited, mild, systemic illness in humans.

HISTORY OF RABIES

Rabies is an ancient disease with references as far back as 2300 BC in the pre-Mosaic Eshnunna Code of Mesopotamia (1). In Greek and Roman times the works of Democritus (460–370 BC), Hippocrates (460–377 BC), Aristotle (384–322 BC), and Celsus (25 BC-50 AD) made reference to rabies in both humans and animals (2). Celsus described human rabies and used the term *hydrophobia*, which is derived from the Greek words meaning fear of water, and at that time he recognized that that the saliva of the rabid animal contained the poisonous agent (2).

In 1769, the pathologist John Morgagni (1735–1789) wrote that rabies virus "does not seem to be carried through the veins, but by the nerves, up to their origins" (3), indicating his insight into a mechanism of neural spread by rabies virus at a very early time before experimental studies had been first performed. In the early 19th century Zinke (1771–1813) discovered that the infectious agent causing rabies was transmitted in the saliva by experimentally painting saliva from a rabid dog into incisions made in healthy animals (4). In 1879, Galtier (1846–1908), who was a professor at a veterinary school in Lyon, France, used rabbits in his experimental work on rabies and he found that using rabbits was technically much less difficult and dangerous than using dogs and cats (5). Subsequently, Louis Pasteur (1822–1895) also used this experimental rabbit model of rabies. Pasteur transmitted rabies virus by inoculating CNS tissues of rabies animals into the brains of other animals, and he observed that sequential brain passages led to attenuation of the agent after peripheral inoculation (6). In 1885, Pasteur successfully immunized a 9-year-old boy, Joseph Meister, who had been severely bitten by a rabid dog, with a series of inoculations of infected rabbit spinal cord tissues that he had partially inactivated by subjecting them to variable periods of desiccation (7). Joseph Meister did not develop rabies and subsequently many people with rabies exposures were immunized with nervous system vaccines in Paris and other locations throughout the world.

In 1903, Adelchi Negri (1876–1912) described eosinophilic cytoplasmic inclusions in infected neurons, which are now called Negri bodies (8). Negri bodies have proven to be useful in making a pathological diagnosis of rabies. In 1958, Goldwasser and Kissling (9) used fluorescent antibody staining in order to demonstrate rabies virus antigens in tissues, which proved to be much more sensitive for rabies diagnosis and also

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for the development of early rabies pathogenesis studies in animals, which were performed by Richard Johnson (10) and Frederick Murphy (11, 12) and their colleagues.

VIROLOGY OF THE RHABDOVIRUSES

The *Rhabdoviridae* is a virus family (along with four others) in the viral order *Mononegavirales*, which are nonsegmented negative strand (anti-sense) RNA viruses. The *Rhabdoviridae* consist of both animal (vertebrates and invertebrates) and plant viruses in 11 different genera. The name rhabdovirus was derived from the Greek "rhabdos," meaning rod. However, the viruses actually have a bullet-like morphology (13), which can be demonstrated by electron microscopy. The *Lyssavirus* genus contains 14 species, including rabies virus that causes infections in humans and other mammals. The *Vesiculovirus* genus has another well-known species, VSV (Alagoas, Indiana, and New Jersey) that causes infections in animals and only incidentally in humans after contact with infected animals. It has been recognized that rabies virus and VSV have many structural features in common. Rhabdoviruses are RNA viruses capable of rapid evolution because of the high error rate of their RNA polymerases with the lack of proofreading activity.

Lyssaviruses

Rabies virus belongs to genotype 1 of lyssaviruses, which includes wild-type or street rabies virus strains and also laboratory-adapted strains, including vaccine strains. The sequence of rabies virus consists of 11,932 nucleotides that code for five viral proteins: nucleocapsid protein (N), matrix protein (M), phosphoprotein (P), glycoprotein (G), and large polymerase protein (L) (14) (Figure 1). A ribonucleoprotein (RNP) core of the virion is formed by helical genomic RNA associated with the N, P, and L proteins. The RNP serves as a functional template for viral transcription and replication. The G and M proteins are associated with a lipid-bilayer envelope surrounding the RNP core. The G protein forms spike-like projections on the surface of the viral envelope and serves as the major surface antigen of rabies virus and binds viral neutralizing antibodies and is important for stimulation of an immune response.

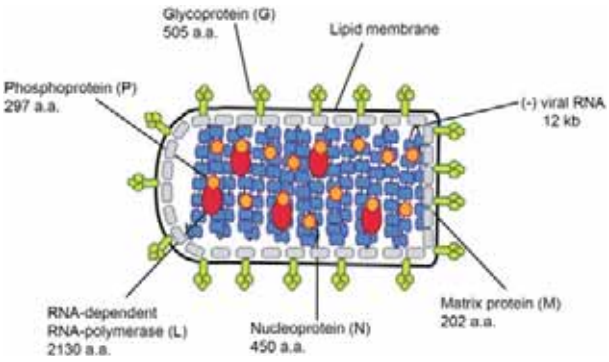

FIGURE 1 Schematic representation of the rabies virus particle. Viral proteins: N for nucleoprotein, P for phosphoprotein, M for matrix protein, G for glycoprotein, and L for large protein; their length in amino acids are indicated. The viral membrane is covered by the glycoprotein G, whereas M is located beneath the membrane. N is bound to the genomic RNA and together with P and L forms the ribonucleoprotein, which constitutes the active viral replication unit. (Reproduced with permission from Albertini AAV et al.: Rabies virus transcription and replication, in Research Advances in Rabies, Alan C. Jackson (ed), Advances in Virus Research 79:1–22, 2011. Copyright Elsevier.)

In recent years, some of the nonrabies virus lyssaviruses have been recognized to very rarely cause human disease, which is clinically and pathologically indistinguishable from rabies, include Mokola virus (genotype 3), Duvenhage virus (genotype 3), European bat lyssavirus 1 (genotype 5), European bat lyssavirus 2 (genotype 6), Australian bat lyssavirus (genotype 7), and Irkut virus (genotype pending) (15).

Vesiculoviruses

VSV is the prototype rhabdovirus and more research has been done on its molecular virology and biochemistry than any other member of the *Rhabdoviridae*. VSV has the same five genes (N, M, P, G, and L) as rabies virus in its genome organization and its virion structure, and the replication cycles are also similar and will not be addressed further. Two serotypes of VSV, New Jersey and Indiana (type 1), cause outbreaks in animals in the United States and there are additional VSV serotypes in Latin America (e.g., Algoas, Cocal, and Piry).

PATHOGENESIS

Lyssavirus Infections

Except under unusual circumstances, rabies virus is transmitted to humans and animals in the saliva via an animal bite. However, a scratch or abrasion with salivary (or brain

TABLE 1 Cases of human rabies associated with organ transplantation in the USA (19–21), Germany (22), and Kuwait/Saudi Arabia (22a). (Adapted with permission from Jackson AC: Human disease, in Rabies: scientific basis of the disease and its management, Third Edition, edited by AC Jackson, 2013, Elsevier Academic Press, Oxford, UK, pp 269–298. Copyright Elsevier.)

	Sex/Age	Organ transplanted	Onset of clinical rabies post-transplantation
Donor in USA	male/20	–	–
Recipient 1	male/53	liver	21 days
Recipient 2	female/50	kidney	27 days
Recipient 3	male/18	kidney	27 days
Recipient 4	female/55	iliac artery segment (for a liver)	27 days
Donor in Germany	female/26	–	–
Recipient 1	female/46	lung	6 weeks
Recipient 2	male/72	kidney	5 weeks
Recipient 3	male/47	kidney and pancreas	5 weeks
Donor in USA	male/20	–	–
Recipient	male/49	kidney	18 months
Donor in Kuwait	male/28	–	–
Recipient 1 in Kuwait	female	kidney	8–9 weeks
Recipient 2 in Kuwait	unknown	kidney	9–10 weeks
Recipient 3 in Saudi Arabia	unknown	heart	unknown
Recipient 4 in Saudi Arabia	male	liver	unknown

or spinal cord tissue) contamination can also result in viral transmission. Aerosol transmission in laboratory accidents (16, 17) and in a cave containing millions of bats (18) have also been documented, but only occur very rarely. Organ (Table 1) and tissue (corneal and vascular conduit) transplantation are also well-documented causes of transmission of rabies virus in humans, which account for a total of 16 well-documented cases (19–22).

Experimental studies in animal models of rabies have provided much information about the events that occur in rabies pathogenesis (Figure 2). The incubation period is longer than many other infections and typically lasts approximately 20 to 90 days after the time of the exposure (e.g., bite), although it may vary from just a few days to over a year or more. Based on animal studies, rabies virus is thought to remain close to the site of viral entry during most of this incubation period (24). After an exposure involving muscle, rabies virus is known to bind to nicotinic acetylcholine receptors (25) that are located in the postsynaptic membrane of the neuromuscular junction. After the virus crosses the synaptic cleft, it spreads centripetally towards the spinal cord in motor nerve fibers of peripheral nerves by retrograde fast axonal transport (26). Bats cause more superficial exposures than larger animals and their bites typically involve cutaneous and subcutaneous tissues, but experimental studies have not

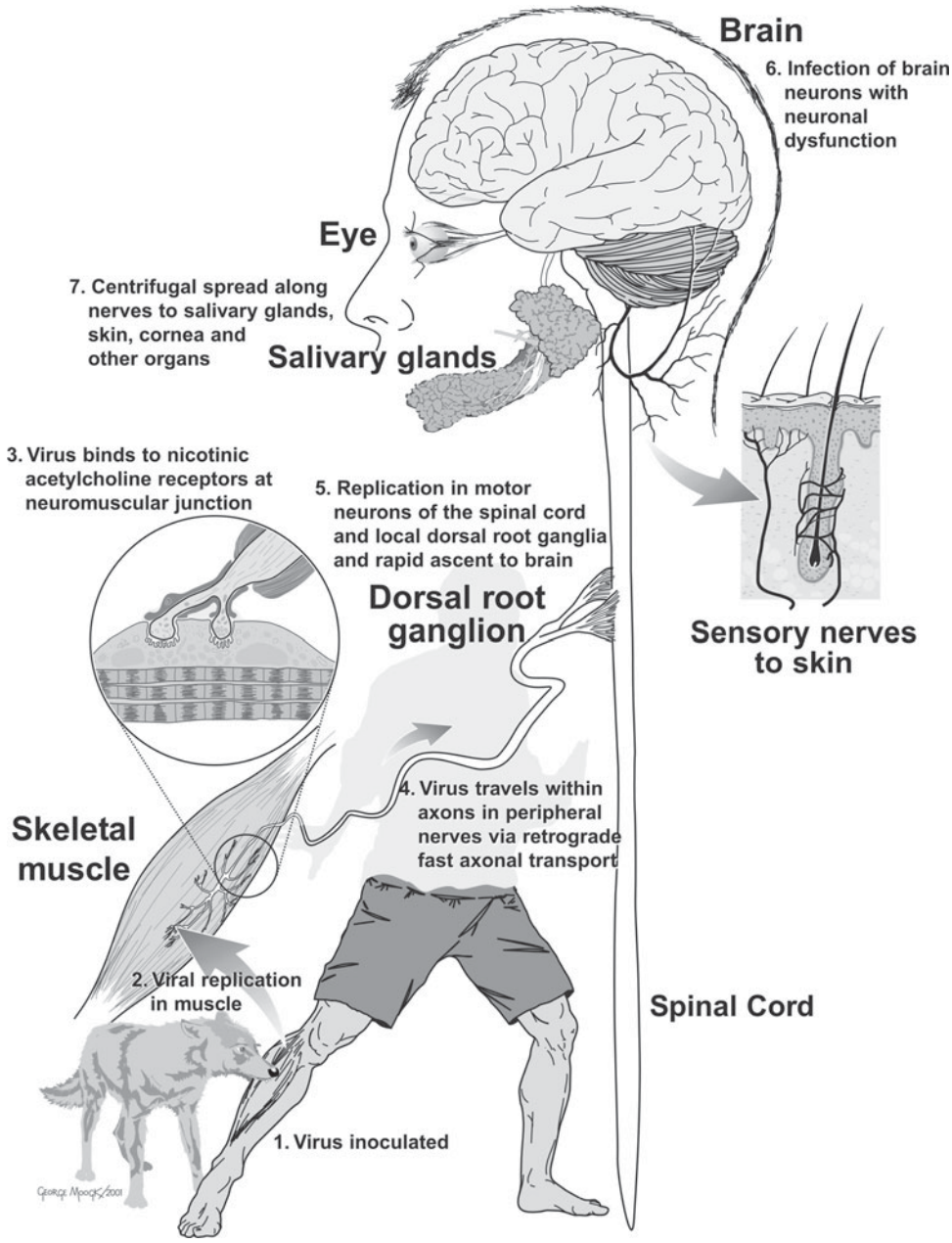

FIGURE 2 Schematic diagram showing the sequential steps in the pathogenesis of rabies after an animal bite/peripheral inoculation of rabies virus. (Reproduced with permission from Jackson AC, Fu ZF: Pathogenesis, in Rabies: scientific basis of the disease and its management, Third Edition, edited by AC Jackson, 2013, Elsevier Academic Press, Oxford, UK, pp 299–349. Copyright Elsevier.)

yet been performed to elucidate the detailed pathways of viral spread in animal hosts.

After infecting spinal cord neurons, rabies virus spreads widely within axons of the CNS by fast axonal transport along neuroanatomical connections. After CNS infection is established, there is centrifugal spread of rabies virus to multiple organs along sensory and/or autonomic nerves. In rabies vectors, viral spread to the salivary glands is important and saliva is secreted containing high titer infectious rabies virus, which is important for transmission to new hosts via bite exposures. Viral spread also occurs to multiple extraneural organs, including the heart (resulting in myocarditis in some cases), adrenal medulla, gastrointestinal tract, and skin (skin biopsy is used for antemortem rabies diagnosis in humans) (27).

Vesiculovirus Infections

Relatively little is known about the pathogenesis of vesiculovirus infections. Intralingual inoculation of VSV into cattle or other animals results in vesicle formation at the site of inoculation (28), whereas intramuscular inoculation typically results in inapparent infection and immunity (29).

PATHOLOGY OF RABIES

Despite the severe clinical disease in rabies, the neuropathological changes are relatively mild. Rabies virus prominently and predominantly infects neurons, although infection of glial cells has been recognized (30). There are mild inflammatory changes, but there are relatively few degenerative neuronal changes. Characteristic microscopic features of rabies encephalomyelitis include mild mononuclear inflammatory changes involving the leptomeninges, perivascular regions, and the CNS parenchyma. Microglial nodules described by Babes (31) are observed in the parenchyma and consist of activated microglia and monocytes, which are called Babes' nodules. Neuronophagia may be observed with accumulations of activated microglia/macrophages in the process of phagocytosing degenerating or dying neurons (32), but degeneration of numerous neurons is not a typical pathological feature of rabies. Negri bodies are characteristic eosinophilic inclusions that are observed in some infected neurons (Figure 3), which were described by and named after Adelchi Negri (8, 33). Electron microscopy has demonstrated that Negri bodies are composed of large aggregates of granulofilamentous matrix material and variable numbers of viral particles (32). Both clinical and pathological findings in fatal human cases due to nonrabies lyssavirus infections are the same as in rabies due to rabies virus (genotype 1) infection.

EPIDEMIOLOGY

Lyssavirus Infections

About 99% of global human rabies cases are a consequence of transmission from dogs due to the presence of endemic dog rabies. There are approximately 59,000 human deaths per year related to endemic canine rabies (34) and the greatest burden of human disease occurs in Asia and Africa. The means of controlling endemic dog rabies are very well established (35). However, for a variety of complex economic, cultural, and political reasons, endemic dog rabies persists in many countries and is an ongoing threat to humans living or visiting these regions, who are at risk of transmission via dog bites. In other countries (e.g., North America) rabies is endemic in wildlife and this poses the main risk for transmission of rabies virus. Rabies virus variants can be identified with molecular techniques, including

FIGURE 3 Three large Negri bodies in the cytoplasm of a cerebellar Purkinje cell from an 8-year-old boy who died of rabies after being bitten by a rabid dog in Mexico. (Reproduced with permission from AC Jackson, E Lopez-Corella, N Engl J Med 335:568, 1996. Copyright Massachusetts Medical Society.)

monoclonal antibody characterization and reverse transcription polymerase chain reaction (RT-PCR) amplification with sequencing, which typically reveals the probable source of the infection when it is not readily apparent.

Because of their threat to human health, bats are the most important wildlife rabies vectors. In the United States, endemic bat rabies is present in every state except Hawaii (Figure 4A). Because of the small size of bats, their bites may not be readily recognized and, consequently, there is no opportunity for the initiation of highly effective preventive measures.

Patients infected by bat rabies virus variants via unrecognized bites may not even be aware that they have had contact with bats. A bat rabies virus variant associated with both silver-haired bats and tricolored bats is most frequently associated with human rabies in the United States and Canada. A variant associated with Brazilian (Mexican) free-tailed bats is the second most common bat variant associated with human rabies cases in the United States. Although big brown and little brown bats are often found in North American houses and are commonly found to be infected with rabies virus, the associated bat rabies virus variants are only infrequently responsible for human cases of disease.

Terrestrial animals that are vectors of rabies in North America include raccoons, skunks, and foxes (36) (Figure 5). Raccoon rabies is endemic along the entire eastern coast of the United States (Figure 4B). In the 1940s, raccoon rabies was initially present in Florida and over a period of decades it gradually spread north and the first incursion into Canada occurred in Ontario in 1999. There are only two documented cases of human rabies case due to raccoon rabies virus variants (21, 37), likely because raccoon exposures are usually recognized. Skunk rabies is present in the midwestern United States, the prairie provinces of Canada (Saskatch-

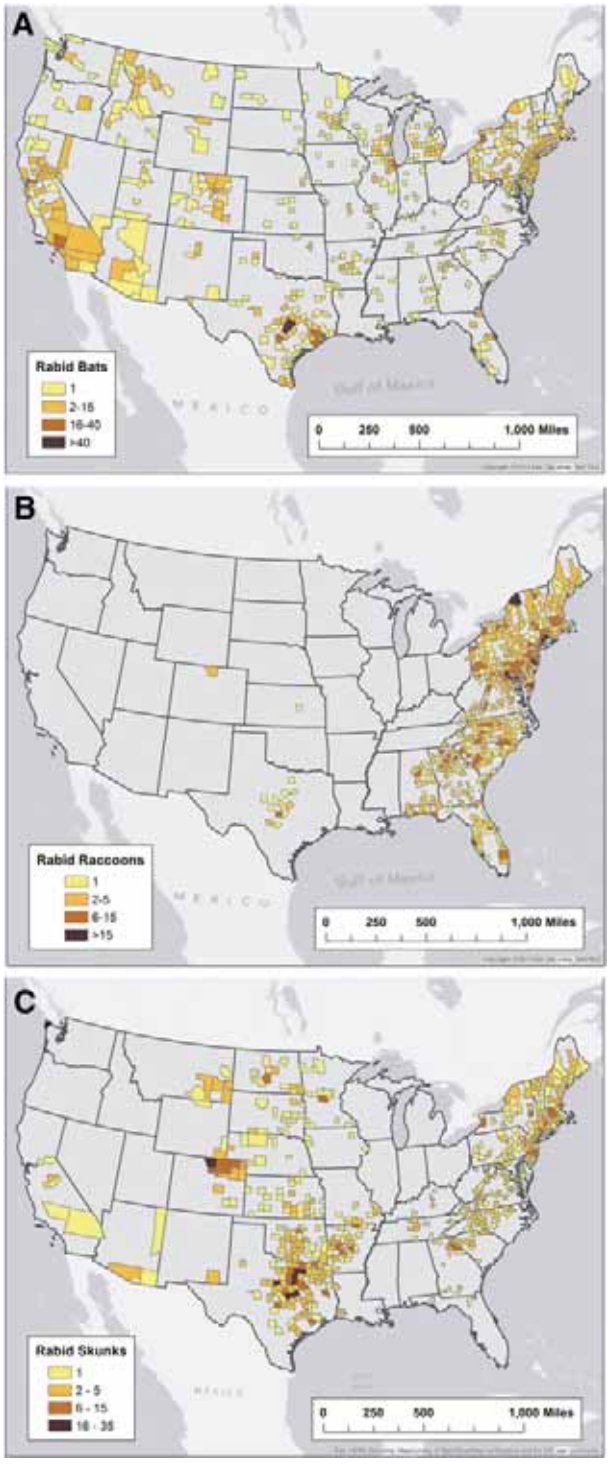

FIGURE 4 Reported cases of rabies involving bats (A), raccoons (B), and skunks (C) by county, 2013. Adapted from JL Dyer et al., J Am Vet Med Assoc 245:1111–1123, 2014. (Centers for Disease Control and Prevention.)

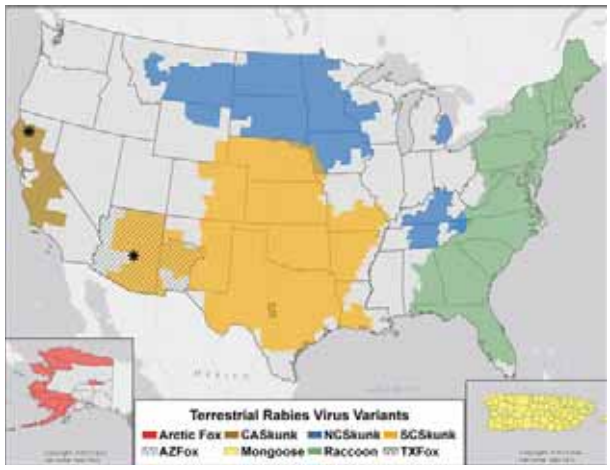

FIGURE 5 Distribution of the major rabies virus variants among wild terrestrial reservoirs in the United States and Puerto Rico, 2009 to 2013. * Potential host shift event. AZ = Arizona. CA = California. NC = North central. SC = South central. TX = Texas. Reproduced from JL Dyer et al., J Am Vet Med Assoc 245:1111–1123, 2014. (Centers for Disease Control and Prevention.)

ewan and Manitoba), and California (Figure 4C). Fox rabies is now uncommon in North America and it has been well controlled with oral vaccination programs in Ontario (red fox) and Texas (gray fox), and also in Europe. Companion animals, especially dogs and cats, are also at risk of developing rabies transmitted from wildlife vectors and, consequently, they may then pose a danger to humans.

Vesiculovirus Infections

Mechanisms involved in VSV transmission are not well understood. VSV needs to penetrate the skin or mucous membranes via an injury or an insect vector (e.g., mosquitoes and sand flies). VSV is a viral disease of cattle, horses, pigs, and some wild mammals caused by VSV infection. VSV occurs in the Western hemisphere, especially in Latin America. Outbreaks in animals are usually seasonal, typically occur at the end of the rainy season in tropical regions and in late summer in temperate zones. Transmission to humans usually results from direct contact with infected animals, particularly cattle, and also occasionally from laboratory exposures.

CLINICAL FEATURES

Lyssavirus Infections

Typically, the incubation period from the time of the exposure (e.g., bite) until the time of the onset of clinical disease lasts between 20 and 90 days, but may be as short as only a few days or exceed a year or more. There is documentation of cases with incubation periods longer than a year, including one as long as 6 years (38). Prodromal symptoms in rabies are nonspecific and include fever, chills, malaise, fatigue, insomnia, anorexia, headache, anxiety, and irritability. They may last for up to 10 days prior to the onset of neurological symptoms. The earliest neurological symptoms of rabies include paresthesias, pain, and pruritus at or close to the site of exposure. They are likely due to infection and inflammatory changes involving local sensory ganglia (e.g., dorsal root ganglia or cranial sensory ganglia). The wound may have completely healed by the time these symptoms develop, or the site of exposure may be unknown (e.g., no recognized animal bite). There are two clinical forms of disease in rabies: encephalitic rabies (in 80% of cases) and paralytic rabies (in 20% of cases). It is likely that the main burden of the

infection in encephalitic rabies involves the brain, whereas in paralytic rabies the main burden likely involves the spinal cord, nerve roots, and peripheral nerves. Fever is present in most cases. In encephalitic rabies there may be episodes of generalized arousal or hyperexcitability, which are separated by lucid periods (39). Patients may have aggressive behavior, confusion, and hallucinations. Features of autonomic dysfunction, including hypersalivation, piloerection (gooseflesh), sweating, priapism, and cardiac arrhythmias, are common. Hydrophobia is a very characteristic clinical manifestation of encephalitic rabies, and occurs more frequently with infections due to rabies virus variants associated with dogs than with bats (40). Patients may initially have pain in the throat or have difficulty swallowing. When they attempt to swallow they experience contractions of the diaphragm and other inspiratory muscles, typically lasting for 5 to 15 seconds. Subsequently, this may become a conditioned reflex and the sight, sound, or even mention of water (or other liquids) may trigger the spasms. Aerophobia is the occurrence of these same spasms precipitated by a draft of air on the skin. As the disease progresses there is progressive neurological deterioration with worsening in the level of consciousness to coma and the development of paralysis.

In paralytic rabies there is early prominent weakness that usually initially involves the bitten extremity and progresses to involve the other extremities and facial muscles. Sphincter involvement, pain, and sensory disturbances also occur. Hydrophobia is unusual in paralytic rabies, although weakness of bulbar and respiratory muscles also develops. Patients with paralytic rabies later develop neurological deterioration with progression to coma, and they typically survive longer than patients with encephalitic rabies.

Medical complications are common in rabies patients treated aggressively in a critical care unit. Cardiac and respiratory complications are common. Cardiac disorders include heart failure, hypotension, a variety of arrhythmias, and cardiac arrest. Both cardiac ganglia and the myocardium may become infected with rabies virus, and in some cases there is an associated myocarditis (41–43). Respiratory complications include hyperventilation, hypoxemia, respiratory depression with apnea, atelectasis, and aspiration pneumonia (44). Hyperthermia or hypothermia may occur, likely secondary to hypothalamic infection. Endocrine complications include inappropriate secretion of antidiuretic hormone and diabetes insipidus (44, 45). Multiple organ failure commonly occurs in patients treated aggressively in critical care units.

Vesiculovirus Infections

VSV in animals is associated with severe vesiculation and ulceration of oral tissues, teats, and feet. Most infections are subclinical. In humans, early conjunctivitis is followed by an acute influenza-like illness with fever, chills, nausea, vomiting, headache, retrobulbar pain, myalgias, substernal pain, malaise, pharyngitis, and lymphadenitis. Small vesicular lesions may be present on the buccal mucosa or on the fingers. Encephalitis is very rare. Typically, the illness usually lasts 3 to 6 days and there is complete recovery. Subclinical infections are common.

DIFFERENTIAL DIAGNOSIS

Lyssavirus Infections

The diagnosis of rabies may be difficult without a history of animal exposure, particularly in countries where rabies rarely occurs, because physicians fail to consider the diagnosis due to a lack of familiarity with the disease, even in typical cases. Physicians may not ask about animal exposures and the patient may not recall an exposure or may not be able to provide this information at the time of presentation. In early phases, encephalitic rabies may be misdiagnosed as a psychiatric disorder, whereas paralytic rabies may be misdiagnosed as Guillain-Barré syndrome. Rabies hysteria is a conversion disorder (somatoform disorder) that may occur as a psychological response to the fear of developing rabies (46). It is characterized by a shorter incubation period than rabies, aggressive behavior (not common in humans), inability for the patient to communicate, and a long clinical course with recovery.

Other viral encephalitides may show behavioral changes with fluctuations in the level of consciousness. Hydrophobic spasms are not observed, and the presence of brainstem signs is unusual in conscious patients in most of the other viral encephalitides. Herpes simiae (B virus) encephalomyelitis, which is transmitted by monkey bites, is usually associated with a shorter incubation period and recovery may occur (47). Tetanus has a shorter incubation period (typically 3 to 21 days) than rabies and is characterized by sustained muscle rigidity involving paraspinal, abdominal, masseter (trismus), laryngeal, and respiratory muscles with superimposed brief recurrent muscle spasms (48). In tetanus, consciousness is preserved, there is no cerebrospinal (CSF) pleocytosis, and the prognosis is much better than in rabies. In Africa, rabies is commonly misdiagnosed as cerebral malaria (49). Anti-*N*-methyl-D-aspartate receptor (anti-NMDA) encephalitis occurs in young patients (especially females) and is characterized by behavioral changes, autonomic instability, hypoventilation, and seizures, and it has recently been recognized that this autoimmune disease rivals viral etiologies as a cause of encephalitis (50). Postvaccinal encephalomyelitis is an important differential diagnosis in patients immunized with a vaccine derived from neural tissues (e.g., Semple vaccine), which is currently used in only a few resource poor countries. Patients with paralytic rabies may resemble the Guillain-Barré syndrome and the pathological features may be also be similar (51). Local symptoms at the site of the bite, piloerection, early or persistent bladder dysfunction, and fever are all more suggestive of paralytic rabies.

Vesiculovirus Infections

VSV in animals may be clinically indistinguishable from the more serious foot-and-mouth disease and vesicular exanthema. In humans, VSV infection presents as a nonspecific, mild, and self-limited systemic viral illness. The presence of vesicular lesions (buccal mucosa or fingers) and exposure to animals are important clinical clues to the etiology.

INVESTIGATIONS

Lyssavirus Infections

Routine blood tests and CT head scans are typically normal in rabies. MRI may be normal or show signal abnormalities in the brain, spinal cord, and/or nerve roots/plexuses, but these findings are not specific for rabies and the main usefulness of MRI is to exclude other diagnostic possibilities (52). CSF analysis usually shows a mononuclear pleocytosis with a cell count of less than 100 cells per μl. Serum neutralizing antirabies virus antibodies may develop in unvaccinated patients, but may not appear for a week or more during the clinical course of disease, and some patients never

develop antibodies prior to death. Neutralizing antirabies virus antibodies may also develop in the CSF, whereas CSF antibodies are not present in vaccinated patients who do not have rabies encephalitis. Specific laboratory tests for confirmation of a diagnosis of rabies include a full thickness skin biopsy taken from the posterior region of the neck at the hairline. Rabies virus antigen may be detected in nerve fibers around hair follicles with direct fluorescent antibody staining. Rabies virus RNA may be detected in fluids or tissues using RT-PCR amplification. Saliva is the most useful specimen for the detection of rabies virus RNA using RT-PCR. RT-PCR can also be used on skin biopsies (53) and CSF, but is much less sensitive on CSF. A negative laboratory test for rabies never excludes rabies unless performed on brain tissues, and the tests may need to be repeated for diagnostic confirmation of a rabies diagnosis. Brain tissues are only very rarely obtained by biopsy antemortem, but are routinely evaluated postmortem by direct fluorescent antibody staining and by culture techniques.

Vesiculovirus Infections

A serologic diagnosis can be made on the basis of a rise in titer of either complement-fixing or neutralizing antibodies, RT-PCR for viral RNA has recently been described on clinical samples (54).

RABIES PREVENTION

After recognized exposures, rabies can be very effectively prevented, whereas unrecognized exposures (e.g., bat bite) allow no opportunity for intervention. Detailed guidelines that are periodically updated are available from the Centers for Disease Control and Prevention (55) and from the World Health Organization (56) on the Morbidity and Mortality Weekly Report (http://www.cdc.gov/mmwr/) and World Health Organization (http://www.who.int/en/) websites, respectively. Algorithms have been developed concerning the decision-making process for initiation of postexposure rabies prophylaxis (57). The first step is to determine whether there is a real risk of rabies virus transmission. This depends on obtaining: the details of the exposure, the species of animal involved, and also on the local epidemiological situation. Advice from local public health officials can be very helpful in making a determination of whether postexposure rabies prophylaxis measures should be initiated. Laboratory testing on brain tissues from an animal is needed in order to make a definitive diagnosis of rabies, which is usually performed using an antigen detection method by the fluorescent antibody technique. If a dog, cat, or ferret remains healthy for a 10 day period after an exposure, then a conclusion can confidently be made that rabies virus transmission did not occur during the exposure because the brainstem infection associated with the salivary excretion of infectious virus did not progress to overt clinical signs within the period. Unwanted animals may be killed and the brains tested without an observation period. Other animal species must not be observed after an exposure because there is uncertainty about the period of time for clinical disease to develop and the period may be much greater than 10 days. If an animal escapes after an exposure, then the animal should be considered rabid unless information from public health officials indicates that this is unlikely. Current recommendations indicate that the physical presence of a bat may warrant postexposure prophylaxis when a person such as a small child or sleeping adult is unable to reliably report contact that could have resulted in a bite (55). In light of the low risks and high costs, recommendations for bedroom exposures to a bat while sleeping and without known physical contact have been questioned (58) and require further expert consideration.

Postexposure rabies prophylaxis in previously unvaccinated persons includes wound cleansing and active immunization with rabies vaccine and passive immunization with human rabies immune globulin (HRIG). All animal bite wounds should be thoroughly cleaned with soap and water and, if available, a virucidal agent (e.g., povidine) should be used to irrigate the wounds. In the United States, four doses of rabies vaccine, which was recently reduced from five doses, are recommended on days 0, 3, 7, and 14 (59). Each 1.0 ml dose of vaccine should be given intramuscularly in the deltoid muscle. Two rabies vaccines are currently licensed in the United States and Canada: purified chicken embryo cell vaccine (PCECV) (RabAvert) and human diploid cell vaccine (Imovax). Pregnancy is not a contraindication for immunization. Local and mild systemic adverse effects are common. Local reactions include pain, erythema, edema, and pruritus; systemic reactions include fever, myalgias, headache, and nausea. Antiinflammatory medications and antipyretics may be used, but immunization should not be discontinued. The dose of HRIG is based on weight (20 IU per kg) and HRIG should be infiltrated into and around the wound and the remaining portion of the dose should be given intramuscularly in a different location (e.g., gluteal muscles) than where the vaccine is given. If there are multiple or extensive wounds and a large volume of HRIG is needed for infiltration, then HRIG may be diluted as required for satisfactory infiltration of all the wounds. HRIG should not be given later than 7 days after the first dose of rabies vaccine. Adverse effects of HRIG include local pain and low-grade fever. If HRIG is not available, then purified equine rabies immune globulin, which is much less expensive and more readily available in some rabies endemic countries (e.g., Thailand), may be used in the same manner at a dose of 40 IU/kg.

In persons at risk of rabies exposure, including laboratory workers, veterinarians, and travelers to places with endemic dog rabies (e.g., Asia and Africa), preexposure rabies immunization should be considered. Three doses of vaccine are given on days 0, 7, and 21. When prolonged protection is needed, booster doses of rabies vaccine can be given periodically as required, based on a serum neutralizing antirabies antibody titer. The perceived risk of exposure may determine the frequency of antibody testing (e.g., every six month to every two years). After a rabies exposure in preimmunized individuals, in addition to wound cleansing, two doses of rabies vaccine should be given on days 0 and 3; HRIG should not be given.

MANAGEMENT OF HUMAN RABIES

Unfortunately, human rabies is virtually always fatal despite aggressive therapeutic attempts at therapy. Most survivors have received rabies vaccine prior to the onset of the clinical disease. The therapeutic options for consideration of an aggressive approach for a patient with rabies were evaluated by an expert group (60). Young and previously healthy patients with an early clinical diagnosis of rabies were felt to be the best potential candidates for aggressive therapy (60). Therapies that were suggested for consideration included rabies vaccine, human rabies immune globulin, monoclonal antibodies (for the future), ribavirin, interferon-α, and ketamine. It was felt that combination therapy might improve efficacy in situations in which specific therapies used

individually had failed in the past, similar to the situation for a variety of infectious (HIV and hepatitis C virus infections) and other noninfectious diseases (e.g., cancer).

A 15-year-old female survived rabies in 2004. She had been bitten on her finger by a bat and did not receive post-exposure prophylaxis therapy (61). About a month after the bite, she came to medical attention with typical clinical features of rabies encephalitis. On arrival to hospital in Milwaukee, Wisconsin neutralizing antirabies virus antibodies were present in both the serum and CSF (initially at titers of 1:102 and 1:47, respectively). Nuchal skin biopsies were negative for rabies virus antigen. Rabies virus RNA was not detected in saliva or in the skin biopsies using RT-PCR. She was intubated, and treated with a drug-induced coma, which included the noncompetitive *N*-methyl-D-aspartate (NMDA) antagonist ketamine at 48 mg/kg/day as a continuous infusion, and intravenous midazolam for 7 days. She was maintained in a burst-suppression pattern on her electroencephalogram and given supplemental phenobarbital as needed. She was also treated with antiviral therapy, including intravenous ribavirin and amantadine 200 mg per day administered enterally. She improved and was subsequently discharged from hospital with neurologic deficits and later had further neurologic improvement (62).

This patient is the first documented rabies survivor who had not received any rabies vaccine prior to the onset of clinical rabies. However, it remains uncertain if therapy with one or more specific agents played any significant role in her favorable outcome (63). Since that time, there have been at least 31 cases in which the main components of this ap-

TABLE 2 Cases of human rabies with treatment failures that used the main components of the "Milwaukee protocol." (Updated from Jackson AC: Therapy in human rabies, in Research Advances in Rabies, Alan C. Jackson (ed), Advances in Virus Research 79:365 – 375, 2011. Copyright Elsevier.)

Case no.	Year of death	Age and sex of patient	Virus source	Country	Reference
1	2005	47 male	kidney and pancreas transplant (dog)	Germany	(22)
2	2005	46 female	lung transplant (dog)	Germany	(22)
3	2005	72 male	kidney transplant (dog)	Germany	(22)
4	2005	unknown	dog	India	(86)
5	2005	7 male	vampire bat	Brazil	[a]
6	2005	20–30 female	vampire bat	Brazil	[a]
7	2006	33 male	dog	Thailand	(87)
8	2006	16 male	bat	USA (Texas)	(88)
9	2006	10 female	bat	USA (Indiana)	(89)
10	2006	11 male	dog (Philippines)	USA (California)	(89, 90)
11	2007	73 male	bat	Canada (Alberta)	(72)
12	2007	55 male	dog (Morocco)	Germany	(91)
13	2007	34 female	bat (Kenya)	The Netherlands	(77)
14	2008	5 male	dog	Equatorial Guinea	(92)
15	2008	55 male	bat	USA (Missouri)	(93, 94)
16	2008	9 female	cat (vampire bat variant)	Colombia	(95, 96)
17	2008	15 male	vampire bat	Colombia	(97)
18	2009	37 female	dog (South Africa)	Northern Ireland	(98)
19	2009	42 male	dog (India)	USA (Virginia)	(99)
20	2010	11 female	cat	Romania	(100)
21	2011	41 female	dog (Guinea-Bissau)	Portugal	(101)
22	2011	25 male	dog (Afghanistan)	USA (Massachusetts)	(102)
23	2012	63 male	brown bat	USA (Massachusetts)	(103)
24	2012	9 male	marmoset	Brazil	(104)
25	2012	41 male	dog (Dominican Republic)	Canada (Ontario)	(105)
26	2012	29 male	dog (Mozambique)	South Africa	(106, 107)
27	2012	58 female	dog (India)	United Kingdom	(108)
28	2013	28 male	dog variant with no known exposure (Guatemala)	USA (Texas)	(109)
29	2013	30 male[b]	dog	China (Taiwan)	(110)
30	2014	24 male	dog	India	(111)
31	2014	male	liver transplant (dog)	Saudi Arabia	(23)

[a]Personal communication from Dr. Rita Medeiros, University of Para, Belem, Brazil.
[b]Personal communication from Dr. Ya-Sung Yang, Tri-Service General Hospital, Taipei, Taiwan. Patient was initially in a vegetative state but died within 6 months while in hospice care.

TABLE 3 Cases of human rabies with recovery.[a] (Adapted from Jackson AC: Therapy of human rabies, in Rabies: scientific basis of the disease and its management, Third Edition, edited by AC Jackson, 2013, Elsevier Academic Press, Oxford, UK, pp 573–587. Copyright Elsevier).

Location	Year	Age of patient	Transmission	Immunization prior to onset	Outcome	Reference
United States	1970	6	bat bite	duck embryo vaccine	complete recovery	Hattwick et al. (71)
Argentina	1972	45	dog bites	suckling mouse brain vaccine	moderate sequelae	Porras et al. (112)
United States	1977	32	laboratory (vaccine strain)	preexposure vaccination	severe sequelae	Tillotson et al. (17, 113)
Mexico	1992	9	dog bites	postexposure vaccination (combination)	severe sequelae[b]	Alvarez et al. (114)
India	2000	6	dog bites	postexposure vaccination (combination)	severe sequelae[c]	Madhusudana et al. (115)
United States	2004	15	bat bite	no postexposure therapy	mild sequelae	Hu et al. (62) and Willoughby et al. (61)
Brazil	2008	15	vampire bat bite	postexposure vaccination	severe sequelae	Ministerio da Saude in Brazil (116)
Turkey	2008	17	dog bites	postexposure vaccination (one dose)	complete recovery	Karahocagil et al. (117)
India	2010	8	dog bite	postexposure vaccination and rabies	immunoglobulin	Netravathi et al. (118)
India	2011	17	dog bite	postexposure vaccination	severe sequelae	de Souza and Madhusudana (119)
Chile	2013	25	dog bite(s)	postexposure vaccination (one dose)	moderate sequelae	Galvez et al. (120)
India	2014	16	dog bite(s)	postexposure vaccination	severe sequelae	Thakur (121)
India	2014	6	dog bites	postexposure vaccination and equine rabies immune globulin	severe sequelae	Karande et al. (122)

[a]Recovery of cases with atypical features of rabies without the development of rabies virus neutralizing antibodies have not been not included because they are likely not cases of rabies (68, 123). A case reported by Rawat and Rao (124) was not sufficiently well-documented for inclusion.
[b]Patient died less than four years after developing rabies with marked neurological sequelae (L. Alvarez, personal communication).
[c]Patient died approximately two years after developing rabies with marked neurological sequelae (S. Mahusudana, personal communication).

proach (the "Milwaukee protocol") were used with fatal outcomes (Table 2) (64). The induction of coma *per se* has no established benefit for the management of infectious diseases of the nervous system, and there is no evidence to date supporting this approach in rabies or other viral encephalitides. For this reason, therapeutic coma should not become a routine therapy for the management of rabies. Recent experimental evidence does not support a mechanism of excitotoxicity in a mouse model of rabies and also in rabies virus infection of cultured neurons, and there was also a lack of efficacy of ketamine therapy in cultured neurons and in the mouse model (65). Even in situations in which there is very strong experimental evidence of excitotoxicity in animal models, such as in stroke, numerous clinical trials in humans have failed to demonstrate efficacy of neuroprotective agents (66). This indicates that an effective neuroprotective effect of a therapy given to a single patient without a credible scientific rationale is highly doubtful. It is likely that this patient would have also recovered with only the supportive therapy.

Neutralizing antirabies virus antibodies are an important marker of an adaptive immune response that is essential for clearance of rabies virus and recovery (67). The presence of serum neutralizing antirabies virus antibodies early in a patient's clinical course probably occurs in less than 20% of patients with rabies and is likely an important factor contributing to a favorable outcome. There have been 11 survivors of rabies who received rabies vaccine prior to the onset of the disease, and only one without vaccine (Table 3). This observation suggests that an early immune response may be important for a positive outcome. Recovery of cases with atypical clinical features of rabies without the development of antirabies virus neutralizing antibodies (68, 69) were unlikely actual cases of rabies and these patients should not be considered survivors.

Bat rabies virus variants are probably less neurovirulent than canine virus variants or other variants that are responsible for most human cases of rabies (70), and there is less chance that human rabies due to canine rabies virus variants will have a favorable outcome than cases caused by bat rabies virus variants. The first well-documented survivor of rabies, who was also infected with a bat rabies virus variant, received rabies vaccine prior to the onset of disease and made an excellent neurological recovery (71). It is unknown if the causative bat rabies virus variant in the Milwaukee case was attenuated and had different biological properties than other isolated variants because no virus was isolated in this case. Diagnostic laboratory tests are usually negative for rabies virus antigen and RNA in fluids and tissues, and brain tissues are not normally tested in most survivors of rabies.

This may reflect effective viral clearance in which centrifugal spread of the infection to peripheral organ sites is reduced or very rapid clearance occurs through immune-mediated mechanisms.

Pathological data from a number of human rabies cases treated with the Milwaukee protocol have demonstrated that the therapy is not effective in clearing rabies virus infection from the brain and from preventing neuronal injury. A case from Edmonton (Canada) was treated with the Milwaukee protocol and after termination of the therapeutic coma, the patient remained in a brain death-like state for approximately four weeks (72). At autopsy, there was complete loss of neurons in the cerebral cortex and positive staining for rabies virus antigen was observed in both brainstem and cerebellar neurons, indicating a failure of clearance of the viral infection from the brain and also failure of protection against neuronal injury and loss (72). In Germany, lung and kidney/pancreas recipients from a rabies virus-infected donor developed rabies and were treated with major components of the Milwaukee protocol, including intravenous midazolam, ketamine, and phenobarbital (in one) (22). One patient died within 2 days, whereas the other survived 64 days after the onset of clinical rabies. At autopsy, the two patients had $1.2 - 2.3 \times 10^9$ RNA copies/mg of CNS tissue, indicating ineffective viral clearance. The longer surviving patient showed viral clearance from systemic organs and peripheral nerves. Hence, the Milwaukee protocol therapy has proved ineffective in promoting viral clearance from the CNS in rabies. It now has become fairly clear that the Milwaukee protocol has no role at all in the management of human rabies. Unfortunately, promotion and repetition of this flawed therapy has likely already impeded progress in the development of new and effective therapies for rabies. A better understanding of basic mechanisms underlying rabies pathogenesis in humans and animals is needed, which may prove to be very helpful in the development of novel therapeutic approaches for the management of this dreaded disease.

NONRABIES VIRUS LYSSAVIRUS INFECTIONS

Nonrabies virus lyssaviruses may cause fatal neurological illness that is clinically and pathologically indistinguishable from rabies. Mokola virus has been isolated from shrews, although the reservoir is unknown. In 1971, a 6-year-old girl died with Mokola virus infection (73); another case with mild illness was more likely due to cross-contamination of specimens in the laboratory (74). The index case of Duvenhage virus infection was transmitted by a bat and occurred in South Africa (75), and two additional cases were recently reported (76, 77). There have been two cases reported due to European bat lyssavirus 1 (78, 79) and another two cases due to European bat lyssavirus 2 (80, 81). In 1996 and 1998, cases due to Australian bat lyssavirus were likely transmitted by an insect-eating bat (82) and a fruit-eating bat (flying fox) (83), respectively, and there was another fatal case in 2013 (84). In 2007, a 20-year-old female died in the Primorye Territory, which is in the Russian Far East, due to Irkut virus, which had been previously isolated from a greater tubenosed bat (85).

Vesiculovirus Infections

Therapy of VSV infection is symptomatic.

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Filoviruses

MIKE BRAY AND DANIEL S. CHERTOW

42

INTRODUCTION

The filoviruses are nonsegmented, negative-sense RNA viruses in the family *Filoviridae*, order *Mononegavirales*. The genus *Marburgvirus* consists of a single species of related viruses, for which bats in Central Africa have recently been found to be a reservoir. The other genus, *Ebolavirus*, contains four species (Zaire, Sudan, Bundibugyo, and Ivory Coast) indigenous to Africa, and a fifth, Reston virus, found in the Philippines. It is likely that the African Ebola species are also maintained in bats, but attempts to recover infectious virus from captured animals have been unsuccessful. Except for the Reston agent, all filoviruses cause severe disease in humans, with fatality rates in outbreaks often exceeding 50%.

The first filovirus was recognized in 1967 when the inadvertent importation of infected monkeys from Uganda to a vaccine laboratory in Marburg, Germany led to an outbreak of fulminant illness in workers who came into contact with the animals or their tissues. The occurrence of fever and bleeding in many of the patients led to the classification of the new "Marburg virus" as an agent of viral hemorrhagic fever, a term first used by Russian scientists in the 1930s to designate certain zoonoses seen in the former Soviet Union. The unique filamentous morphology of the novel agent (Fig. 1) led to the designation of a new virus family. Extensive studies of animals trapped in Uganda failed to identify a natural reservoir.

In 1976, two almost simultaneous epidemics in Sudan and Zaire (the present Democratic Republic of the Congo) revealed the existence of the Ebola viruses. Over succeeding decades, some 25 outbreaks of Marburg or Ebola hemorrhagic fever, ranging in size from single infections to more than 400 cases, occurred sporadically across a region of Central Africa extending from the Sudan and Uganda to Gabon and Angola (Table 1, Fig. 2). Outbreaks were brought to an end through the combined efforts of local health care workers and international medical teams. Clinical and epidemiologic studies have found that the four African filovirus species cause similar syndromes, although mortality varies by species, and that person-to-person spread occurs only through direct contact with blood and other body fluids. In the absence of any vaccines or specific therapy, the medical response was based on identifying and isolating infected persons and their close contacts and providing basic supportive care while waiting for the outbreak to "burn out."

In late 2013, the Zaire species of Ebola virus unexpectedly appeared in West Africa and was carried by chains of person-to-person transmission from a village in rural Guinea to the largest cities of Guinea, Liberia, and Sierra Leone. By mid-2014 the virus had killed more human beings than in all previous outbreaks combined. Efforts to halt the epidemic were hampered by the difficulty of recognizing Ebola patients against a background of malaria and other common febrile diseases and by the limited medical, public health, communications, and logistical resources in three of the world's poorest countries. After a massive international response, including the construction and staffing of many dedicated treatment centers (Fig. 3), the epidemic began to decline. In mid-2015, new cases were still occurring, in several instances because of late sexual transmission from male survivors. WHO declared that Ebola transmission had ceased in all three affected countries by late December 2015, although enhanced vigilance for late-onset cases continued for 90 days (1).

The experience of the West African epidemic has brought about two significant changes in this chapter since the previous edition. The first is a major expansion of the sections devoted to the clinical syndrome and patient care. Before 2014, detailed descriptions of individual patients were limited to the 1967 Marburg outbreak, a few cases treated in South African hospitals, and some accidental laboratory infections, and reports of outbreaks contained only general summary data. The occurrence of more than 25,000 cases in the current epidemic, including more than 20 patients treated in the United States and Europe, has produced more detailed knowledge of the disease and approaches to its management that could scarcely have been imagined a year ago.

The second modification in this chapter since the last edition is a change in terminology: as clinicians in West Africa noted that significant bleeding is actually not a common finding in Ebola virus infection, the name of the syndrome has been changed from "Ebola hemorrhagic fever" to "Ebola virus disease." This does not mean that the clinical syndrome differs from earlier outbreaks. Instead, the realization that coagulopathy is only part of the syndrome and that major hemorrhage is common only in the late stage of fatal cases has permitted clearer observation of other aspects of the disease, especially the profuse vomiting and diarrhea that frequently occur early in the course of illness that may

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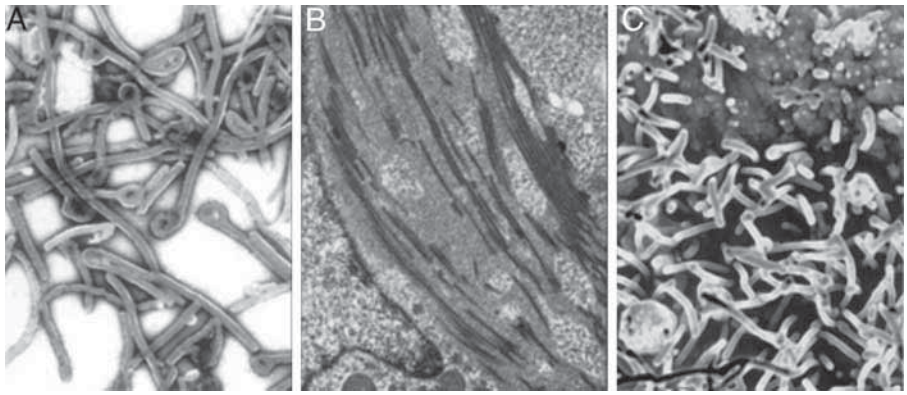

FIGURE 1 A. Negative-contrast electron micrograph (EM) of Ebola virions (magnification, ×17,000). B. Transmission EM of viral nucleocapsids in a cytoplasmic inclusion body. C. Scanning EM of virions budding from the surface of an infected cell. (Courtesy of Tom Geisbert, USAMRIID.)

precipitate hypovolemic shock unless treated by prompt fluid replacement.

The West African epidemic also brought about the accelerated preclinical development and clinical testing of a number of drugs and vaccines, including the administration under emergency use protocols of a number of experimental therapies, principally to accidentally infected Americans and Europeans evacuated to their home countries. Unfortunately, such advanced therapies did not arrive in time to prevent the deaths of nearly 1,000 African doctors and nurses who became infected while caring for patients. It can only be hoped that the experience of this devastating

TABLE 1 Principal outbreaks and individual cases of filovirus disease and known cases and epidemics of filoviral hemorrhagic fever

Virus	Year	Location	Source of infection	Mode of person-to-person spread	No. of cases	CFR (%)
Marburg	1967	Europe	Imported monkeys	Laboratory, hospital	31	23
	1975	South Africa	Unknown	Hospital, direct contact	3	33
	1998	DRC	Unknown	Repeated bat exposures	154	83
	2004	Angola	Unknown	Hospital, direct contact	374	88
	2007	Uganda	Bat exposures	None	4	50
	2012	Uganda	Unknown	Hospital, direct contact	23	65
Ebola Zaire	1976	Zaire	Unknown	Hospital	318	88
	1994	Gabon	Unknown	Hospital/healer	49	65
	1995	DRC	Unknown	Hospital, direct contact	317	77
	1996	Gabon	Dead chimp	Nonhospital contact	37	57
	1996	Gabon	Unknown	Nonhospital contact	60	75
	1996	South Africa	Infected in Gabon	Hospital contact	2	50
	2001	Gabon	Dead chimp	Direct contact	65	82
	2001	Republic of the Congo	Unknown	Direct contact	57	75
	2002	Republic of the Congo	Unknown	Direct contact	143	89
	2003	Republic of the Congo	Unknown	Direct contact	35	83
	2007	DRC	Unknown	Direct contact	264	71
	2008	DRC	? bat exposure	Direct contact	32	44
	2013–15	Guinea, Sierra Leone, Liberia	? bat exposure	Direct contact	>28000	40–60
Ebola Sudan	1976	Sudan	Unknown	Hospital	284	53
	1979	Sudan	Unknown	Hospital	34	65
	2000	Uganda	Unknown	Hospital	425	53
	2004	Sudan	Unknown	Unknown	17	41
Ebola Côte d'Ivoire	1994	Côte d'Ivoire	Necropsy of dead chimp	None	1	0
Ebola Bundibugyo	2007	Uganda	Unknown	Direct contact	149	25

CFR, case fatality rate; DRC, Democratic Republic of the Congo.

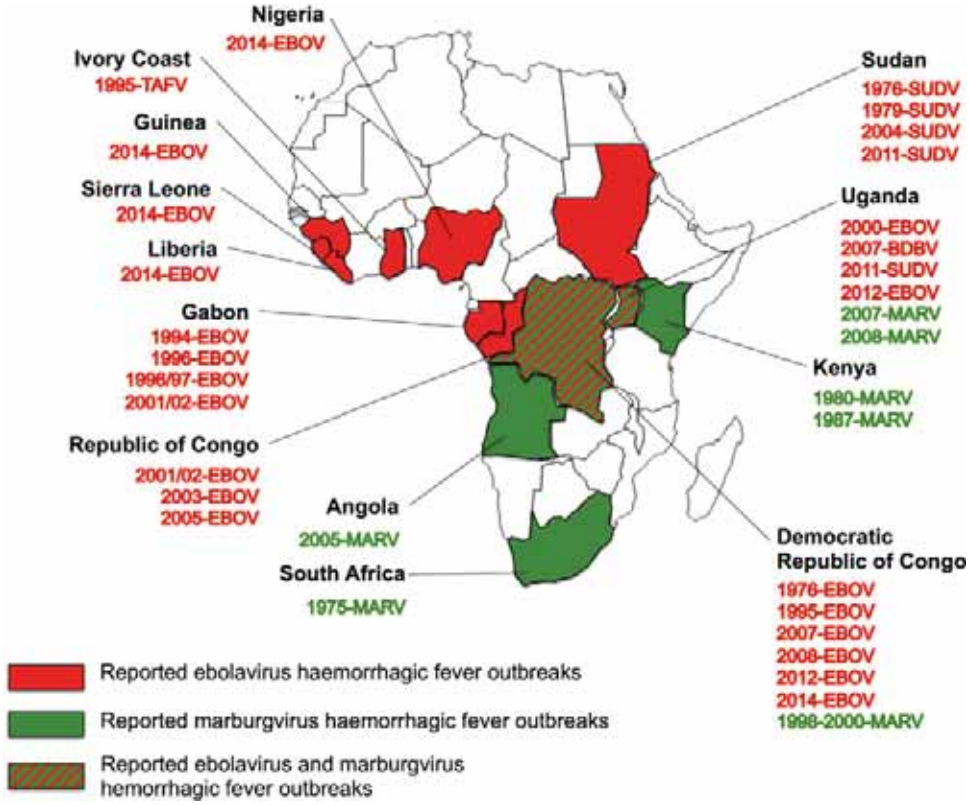

FIGURE 2 Outbreaks of Marburg and Ebola virus disease in Africa, 1976–2014. All Marburg outbreaks have been caused by a single virus species. Most Ebola epidemics have been caused by the Zaire species (EBOV), with the Sudan virus (SUDV) responsible for a smaller number in East Africa. The Bundibugyo (BDBV) virus has been identified only in Uganda and the Ivory Coast virus in a single human infection in the Tai Forest in that country (TAFV). (Courtesy of Eric Leroy, CIRMF, Gabon. Reprinted with permission from Reference 180.)

FIGURE 3 The iconic figure of the West African Ebola epidemic: a health care worker in full personal protective equipment (PPE). The outbreak began in a rural village in Guinea in late 2013, then spread to the largest cities of Guinea, Sierra Leone, and Liberia. By January, 2016, more than 28,000 suspected, probable, and confirmed cases had been reported—more than 10 times the total of all previous Ebola epidemics combined—and 11,000 deaths (181) (Photo provided by Médecins Sans Frontières, used with permission.)

epidemic will lead to the rebuilding and strengthening of the health care system in West Africa so that such a sacrifice will never be repeated.

VIROLOGY

Phylogenetic Classification

The family *Filoviridae* contains three genera, *Marburgvirus*, *Ebolavirus*, and *Cuevavirus*, which are almost identical in genomic organization but differ extensively in nucleotide sequence. A recent phylogenetic analysis has placed the most recent common ancestor of the filoviruses some 10,000 years in the past (Fig. 4). The genus *Cuevavirus* is represented by a single species, *Lloviovirus*, which to date has been detected only as RNA sequences in the tissues of bats in Spain (2).

Although the Marburg viruses show some sequence divergence between the Ravn and Lake Victoria strains, they are still sufficiently closely related that only one species is recognized, and a recombinant vaccine based on the Musoke strain cross-protected macaques against the Angola and Ravn strains (3). In contrast, the genus *Ebolavirus* contains five species: Sudan, Bundibugyo, Reston, Côte d'Ivoire, and Zaire, now designated simply *Ebolavirus*. The earliest evolutionary divergence produced the ancestor of the Reston and Sudan species, while later pathways led to the more closely related Zaire, Bundibugyo, and Tai Forest viruses. The Makona strain of *Ebolavirus* responsible for the West African epidemic has an overall 97% sequence identity with

FIGURE 4 Bayesian coalescent analysis of genomic sequences of the *Filoviridae*, based on representative viruses chosen from each *Ebolavirus* species and a diverse set of *Marburgvirus* isolates. Values at each node represent years since most recent common ancestor, prior to 2007. The tree indicates the early divergence of the genera *Marburgvirus*, *Cueavavirus*, and *Ebolavirus*. Although the Marburg Ravn and Musoke viruses have followed a separate evolutionary course for approximately 1,000 years, they are still more closely related to each other than any two species in the genus *Ebolavirus*. The Sudan and Reston viruses are the most distantly related to other Ebola viruses. (Reprinted from Reference 182 with permission.)

strains isolated earlier in the Democratic Republic of the Congo (DRC) and Gabon (4). The cellular replication mechanisms of Ebola and Marburg viruses differ only in the makeup of the replication complex and production of the surface glycoprotein (GP), as described below.

Composition of Virus

Filoviral virions are long, filamentous structures that measure 80 nm in diameter but can range in length from 800 to more than 10,000 nm. Individual particles appear straight, branched, or U shaped when viewed by electron microscopy (Fig. 1). The outer surface of the virion is made up of a lipid bilayer derived from the host cell, in which trimeric spikes of the virion GP are embedded (Fig. 5A) (5). The central core is composed of a helical nucleocapsid, consisting of the genome and its encapsidating proteins, nucleoprotein (NP), VP35, and VP30. The nucleocapsid is linked to the inner surface of the envelope by the major matrix protein, VP40, and a minor protein, VP24. The RNA-dependent RNA polymerase, or L protein, is also carried within the virion.

Genome

The filoviruses have the longest genomes of the *Mononegavirales*, averaging just over 19 kb. The 3′ and 5′ ends contain conserved, complementary sequences, which are involved in the initiation of transcription and genome replication. The order of the seven genes resembles that of the rhabdoviruses and paramyxoviruses (Fig. 5B). Most genes possess long noncoding regions at their 3′ and 5′ ends. A conserved UAAUU transcriptional signal is present at the 5′ end of start sites and the 3′ end of stop sites.

Major Structural and Regulatory Proteins

Surface Glycoprotein

The surface of filoviral virions bears a single type of GP that is responsible for interactions with host cells and is the primary target for neutralizing antibodies (Fig. 5A). The Marburg virus GP is expressed from a single open reading frame. In contrast, all Ebola virus species encode their surface GPs in two contiguous reading frames, which are offset by a single nucleotide (5). The major product, encoded by the upstream reading frame, is a C-terminally truncated version of GP that lacks a hydrophobic membrane anchor and is secreted from infected cells (sGP). Full-length protein is expressed only when the RNA polymerase "stutters" while transcribing a series of seven U's at a so-called "editing site" and inserts an additional adenosine. This site is absent from

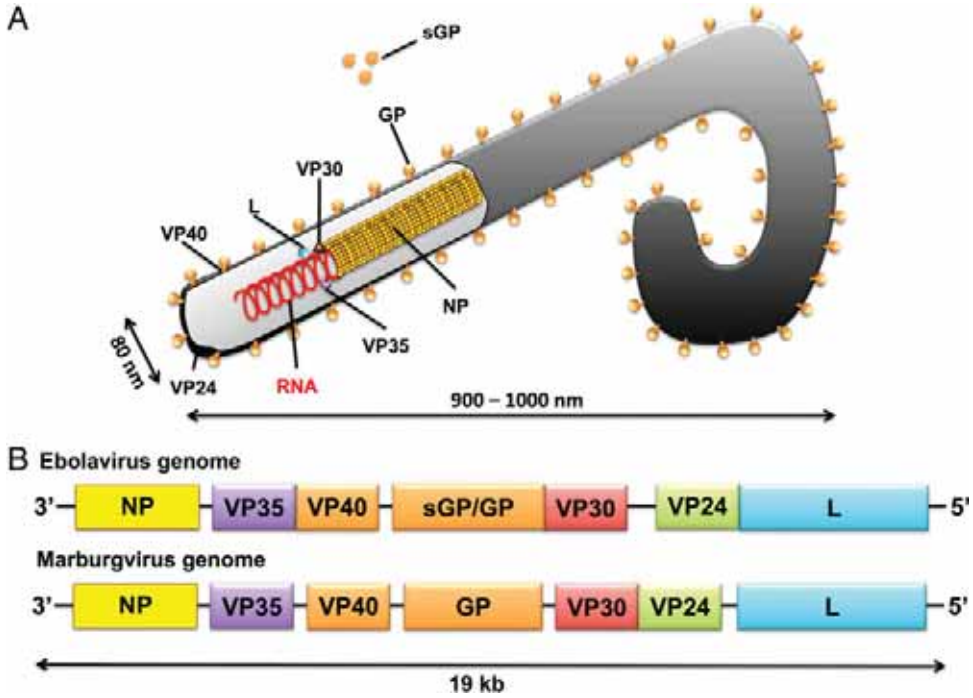

FIGURE 5 A. Structure of a filovirus virion, showing the RNA genome with its associated nucleocapsid proteins, enveloped in a lipid bilayer bearing glycoprotein spikes. NP and VP30 bind to virion RNA to make up the nucleocapsid, and VP35 and the RNA polymerase (L protein) join them in forming a replication complex. Matrix proteins VP24 and VP40 link the nucleocapsid to GP on the inner surface of the envelope. B. Schematic representation of the genomes of Marburg and Ebola viruses. The seven genes are drawn roughly to scale. (Courtesy of Eric Leroy, CIRMF, Gabon. Reprinted from Reference 180 with permission.)

the Marburg virus GP gene. Although it has been argued that sGP somehow contributes to the virulence of the Ebola viruses, its absence from the equally virulent Marburg virus argues against any significant role in pathogenesis.

Both sGP and the full-length molecule undergo extensive N- and O-linked glycosylation in the Golgi apparatus and are then cleaved by a furin-like enzyme into two segments that remain linked by a disulfide bond. The final membrane-bound product ($GP_{1,2}$) is composed of the 140-kDa GP_1, which is responsible for receptor binding and the 26-kDa membrane-anchored GP_2, which contains a hydrophobic stretch of amino acids that fuses with the host cell membrane (6). $GP_{1,2}$ molecules aggregate into homotrimers on the virion surface. Some GP is cleaved by a cellular metalloprotease, tumor necrosis factor-α converting enzyme, and released into the circulation; this "shed GP" has been shown to bind to dendritic cells and macrophages, inducing the secretion of a range of pro- and antiinflammatory cytokines and the expression of cell-surface costimulatory molecules (7).

Matrix Proteins

The matrix protein, VP40, is the most abundant protein in the virion and is the driving force behind the assembly and release of new viral particles from infected cells (6, 8). Like homologous proteins of other viruses, VP40 has evolved to resemble a host cell protein by acquiring a "late domain" sequence that is recognized by elements of the vesicular protein-sorting machinery, particularly Tsg101 and the ubiquitin ligase Nedd4, permitting interactions with microtubules that carry VP40 to the cell membrane. There it associates with cholesterol-rich lipid rafts, which serve as organizing centers for virion formation (9).

Nucleocapsid

The filoviral nucleocapsid is a tubular structure 50 nm in diameter, with a central axial channel (Fig. 5A). Its principal component is the NP, a roughly 100-kDa phosphoprotein whose hydrophobic N-terminal segment binds to genomic RNA. When VP24 and VP35 are coexpressed, structures form that closely resemble nucleocapsids in virus-infected cells. In the presence of VP40, nucleocapsids move to the inner surface of the cell membrane, demonstrating the critical role of that protein in assembling structures required for virion budding.

Nonstructural/Regulatory Proteins

Two filovirus nonstructural proteins make important contributions to virulence through the evasion of innate immune responses. VP35 inhibits cellular recognition of viral dsRNA, thus preventing the initiation of a type I interferon (IFN) response, while VP24 interferes with the transport of phosphorylated STAT1 into the nucleus, so that infected cells do not respond to exogenous type I IFN (10, 11).

Biology

Replication Strategy

The mannose-rich oligosaccharides on the Ebola and Marburg virus surface GPs bind to widely distributed C-type lectins, including DC-SIGN and L-SIGN, but specific entry receptors have not been identified. Following internalization in a macropinosome, GP is "primed" for membrane fusion through cleavage by cathepsins B and L, which remove more than half of the molecule, including the mucin-like domain. Endosomal acidification then leads to triggering of membrane fusion, in a process that requires the Niemann-Pick

(NP) C1 protein; mice lacking NPC1 are solidly resistant to an otherwise lethal Ebola virus challenge (12, 13). Virions enter coated pits and are enveloped by endocytic vesicles; acidification then leads to a conformational change in GP_2, exposing hydrophobic amino acids that carry out membrane fusion, resulting in release of virion contents into the cytoplasm.

Transcription of viral genes is performed in the case of Marburg virus by a replication complex consisting of NP, VP35, and the L protein (polymerase), while that of Ebola virus also includes VP30 (14). Transcription begins at the 3′ end of the genome, producing a leader RNA and seven polyadenylated mRNAs. Production of virion proteins continues until the accumulation of a gene product, probably NP or VP30, triggers the synthesis of complementary copies of the viral genome that serve as templates for replication. As new genomes form, they become encapsidated by NP and other proteins to produce nucleocapsids, which form parallel aggregates in eosinophilic cytoplasmic inclusion bodies (Fig. 1B). Meanwhile, new $GP_{1,2}$ molecules emerging from the Golgi apparatus migrate to lipid rafts, where association with VP40 and nucleocapsids causes assembly and extrusion of new virions (Fig. 1C) (15).

Host Range and Natural Reservoir

Although the first known filovirus outbreak resulted from the introduction of monkeys from Africa into Europe, and the use of virus-infected chimpanzees or gorillas as food by residents of Central Africa has triggered several small epidemics, it is clear that those animals (and in fact no primate species) can be a natural reservoir: Marburg and all five species of Ebola virus have caused severe disease in all primate species tested in the laboratory. However, a recent study found antibodies to Ebola virus in the feces of some 10% of wild apes in the Republic of the Congo, suggesting some animals might survive infection (16). Analysis of wild animal carcasses in Gabon and the Republic of the Congo found PCR evidence of Ebola virus infection in several gorillas and chimpanzees, and in a single duiker (17). It now appears most likely that the filoviruses are maintained in one or more species of small animals that are widely distributed in Central and West Africa, and that wild primates, like humans, become infected through contact with those animals.

Because many single cases and outbreaks over the years have been associated with exposure to bats, they have long been the leading candidates for the natural reservoir. A study following the 1995 Kikwit Ebola outbreak provided support for this hypothesis: inoculation of the causative agent produced persistent viremia but no visible illness in several species of fruit and insectivorous bats (18). More recently, Marburg virus was isolated directly from *Rousettus aegypticus* bats captured in caves in Uganda where human exposures had occurred (19). Studies of these bats in their natural cave environment have revealed periodic shedding of virus by juveniles during the birthing season (20). Captive bats inoculated with a low-passage bat-derived virus became viremic and shed infectious virus in oral secretions (21).

Parallel efforts to identify Ebola virus in bats trapped in a number of sites in Central Africa have detected circulating antibodies and viral RNA in body fluids and tissues but infectious virus has not been recovered (22, 23). People living in rural West Africa are frequently in contact with bats or their excretions, suggesting that the current epidemic arose through exposure of the index case to the body fluids of an infected bat (24). Studies in Gabon and the Republic of the Congo have found that dogs that consume the meat of Ebola-infected animals become seropositive without becoming ill, suggesting they might be a source of human infection (25). There is no evidence of transmission of Ebola or Marburg virus by mosquitoes or other biting arthropods.

Animal Models

Wild-type filoviruses do not cause disease in rabbits, rats, mice, hamsters, guinea pigs, or other common laboratory animals. However, sequential passage of Marburg virus in guinea pigs quickly resulted in the acquisition of lethal virulence for the animals. Ebola Zaire and Ebola Sudan viruses have been adapted to guinea pigs in similar fashion (26, 27). Similarly, a virus that is lethal for adult mice was obtained by serially passaging Ebola virus from the 1976 Zaire outbreak in progressively older suckling mice (28). This variant is more effective at suppressing murine type I interferon responses than wild-type virus; conversely, it appears to be attenuated for nonhuman primates (29). The mouse-adapted virus also causes severe illness in Syrian hamsters, with more features of coagulopathy than are observed in mice (30). Studies of filovirus replication, including antiviral drug testing, have also employed wild-type viruses and knockout mice deficient in type I interferon function, either through loss of the cell-surface IFN-α/β receptor or the cellular STAT1 protein (31, 32).

All filoviruses cause severe disease in captive nonhuman primates that resembles the illness in humans (33). Recent preclinical studies of the efficacy of vaccines, antivirals, and monoclonal antibodies have made use of rhesus and cynomolgus macaques (34–36). Infection of these animals with the Marburg virus or any of the species of Ebola virus produce a similar syndrome, with the onset of lassitude and inactivity 2 to 3 days after infection, followed by the progressive onset of shock. Minor evidence of coagulation defects, such as conjunctival bleeding, are common, but frank bleeding is rare. Changes in coagulation parameters, serum chemistry values, white blood cell and platelet numbers, and circulating cytokine levels resemble those observed in human patients.

Growth in Cell Culture

All studies of live Ebola or Marburg virus replication in cell culture must be performed in Biosafety Level 4 containment. Filoviruses replicate to high titers in many types of cultured cells. Plaque assays for virus titration based on uptake of the vital dye, neutral red, are performed using the Vero E6 cell line. Ebola Zaire and some isolates of Marburg virus cause the death of these cells through necrosis, while the Sudan, Reston, and Ivory Coast strains of Ebola virus are less cytopathic. Syncytium formation is not observed.

Inactivation by Physical and Chemical Agents

Filoviruses are relatively stable in aqueous suspensions and may remain infectious for at least several days in body fluids or when dried on surfaces, or for several weeks at a temperature of 4°C (37, 38). In a study focusing on the persistence of infectious virus in body fluids postmortem, infectious virus was detected in swabs of the oral mucosa of macaques fatally infected with the Zaire or West African Makona strain of Ebola virus for up to a week after death (39). Infectious virus was recovered from blood obtained one week postmortem, and viral RNA was detected for up to nine weeks.

Virus-containing material can be inactivated by treatment with standard bleach solutions, formaldehyde, or phenolic or quaternary ammonium disinfectants. In outbreak settings, bleach diluted in water is most commonly

used for disinfection. For example, in Ebola treatment units (ETUs) operated by Médecins Sans Frontières (MSF), 0.5% active chlorine is used to disinfect personal protective equipment and the environment, and 0.05% active chlorine is used to disinfect body surfaces. A list of EPA registered disinfectants that meet CDC criteria for use against Ebola viruses on hard nonporous surfaces is available (40).

EPIDEMIOLOGY

Single cases or small clusters of filovirus disease have presumably occurred among residents of Central Africa for millennia, but large outbreaks apparently were not seen before the second half of the 20th century. Paradoxically, their appearance was in part a consequence of the construction of clinics and hospitals, which in the absence of proper infection control measures have facilitated person-to-person spread of virus. The vastly larger size of the current West African epidemic does not reflect a change in *Zaire ebolavirus* or its mode of transmission; instead, it has resulted from the introduction of the disease into urban settings in three extremely poor countries that lack the resources needed to control its spread.

Distribution and Geography

Outbreaks of Marburg and Ebola virus disease have occurred over a region of Africa extending roughly 10° to either side of the equator (Table 1, Fig. 2). They have not been restricted to any one type of vegetation pattern or topography. The sporadic and unpredictable occurrence of the disease is consistent with the circulation of the virus among bats with a wide geographic range.

Incidence and Prevalence of Infection

Table 1 lists principal outbreaks of Marburg and Ebola virus disease, beginning with the 1967 Marburg epidemic. Current evidence indicates that Marburg virus and the Zaire and Sudan subtypes of Ebola usually cause severe disease in humans, but mild or even asymptomatic infections may occur. Some serologic surveys beginning in the 1970s reported prevalences of antifilovirus antibodies exceeding 20% among residents of Central Africa, but the absence of any corresponding disease in the same areas suggested a lack of assay specificity (41). However, more recent serosurveys using more specific enzyme-linked immunosorbent assay methods (ELISA) detected IgG antibodies in some 10% of residents of villages in proximity to the 1995 *Zaire ebolavirus* outbreak in the Democratic Republic of the Congo and IgM antibodies to Ebola and Marburg viruses in the sera of 8% to 9% of patients in Sierra Leone being screened for Lassa fever (42). A 1996 study in Gabon found proinflammatory cytokines and specific antibody responses in a small number of family caregivers during an Ebola outbreak in 1996, in the absence of recognized illness (43). Taken together, these studies provide evidence for the occurrence of mild or asymptomatic infections in filovirus-endemic regions of Africa.

Epidemic Patterns

Outbreaks of filovirus disease in humans have followed four different patterns. In the first, infection results from close contact with bats. As noted above, this source of infection has only been proven for Marburg virus, which has infected miners and tourists exposed to large populations of bats in caves in Uganda and the Democratic Republic of the Congo (44).

In the second pattern, which characterized both the original Marburg outbreak in Europe (45) and a number of Ebola epidemics in the border region between Gabon and the Democratic Republic of the Congo, humans have acquired disease through direct contact with filovirus-infected nonhuman primates. Several outbreaks have resulted from the butchering and consumption of chimpanzees or gorillas found sick or dead in the forest, leading to a point-source epidemic (46, 47). The source of infection of great apes has not been proven, but it may result from exposure to infected fruit bats when the animals share food sources (22, 48).

In the third, hospital-based pattern, the disease is recognized when a sick individual is admitted to a hospital lacking basic infection control practices so that the disease spreads to doctors, nurses and other patients. As described below, this was first observed in the Ebola Zaire outbreak in 1976, when the reuse of contaminated syringes simultaneously infected all patients in a mission hospital, and in the 1995 Kikwit epidemic, when a surgical team operated on an unrecognized Ebola patient (49, 50).

In the fourth pattern, exemplified by the current West African epidemic, a filovirus is introduced by an index case infected from an unknown source, then spreads person-to-person within a community when family members and others care for a sick person, prepare a deceased individual for burial, or engage in risk behaviors during the burial process. Because such funeral practices may involve hands-on contact by many persons, they can lead to explosive epidemic spread.

Marburg Virus Disease

The first recognized filovirus outbreak occurred in 1967, when cercopithecus macaques were imported from Uganda to Marburg and Frankfurt, Germany and to Belgrade, Yugoslavia as a source of primary kidney cells for polio vaccine production. Although the animals appeared well on arrival, 25 people who tended them or processed their tissues developed a severe hemorrhagic disease (45). Six doctors and nurses became infected; 7 of the 31 total cases were fatal. Follow-up studies in Uganda failed to reveal the source of the virus. During the next 31 years, only six Marburg virus infections were detected. The first was in a hitchhiker who arrived in South Africa from Rhodesia in 1975; infection also spread to his traveling companion and to a nurse where the two were treated (51). Cases also occurred in 1980 and 1987 in persons who had visited a cave in western Kenya inhabited by large numbers of bats.

The first known Marburg epidemic in Africa was detected in 1998 among men working in bat-infested mines in the Durba/Watsa area of eastern Democratic Republic of the Congo (44). Sequence analysis found that several strains of virus had been introduced on separate occasions into the affected community, suggesting repeated exposure to the virus-containing excreta of a mixed population of bats. In 2007, a cluster of cases occurred in Uganda in miners exposed to bats. In separate incidents in the following years, an American and a Dutch tourist who entered a bat-infested cave developed Marburg virus disease; one died of the disease, while the other recovered and was diagnosed retrospectively (52).

The largest epidemic of Marburg virus disease to date occurred in 2005 in Angola, when the agent was somehow introduced into the pediatric ward of Uige provincial hospital, causing a wave of lethal illness among the young patients, possibly through the use of contaminated transfusion equipment. The epidemic then spread to nurses, doctors, and family caregivers and into the surrounding community (53).

Ebola Virus Disease

Zaire ebolavirus

The first recognized outbreak occurred in June, 1976 at a missionary hospital in Yambuku, close to the Ebola River in northern Zaire (the present Democratic Republic of the Congo) (49). It began when an infected person was admitted to the hospital, and rapid nosocomial spread took place through the reuse of unsterilized glass syringes. The epidemic was further amplified when doctors, nurses, and family members also became infected, but it eventually "burned out" without outside intervention. Another hospital-based outbreak in 1995 in Kikwit, Democratic Republic of the Congo began when an individual with abdominal pain and bloody diarrhea underwent surgery, spreading infection to the operating team (50). The causative agent proved to be almost identical in sequence to that which caused the 1976 epidemic. The epidemic spread as hospital staff and family members came into direct contact with patients; it ended when local and international health workers established an isolation center and instituted rigorous infection control measures.

Over the following decade, additional epidemics occurred in the border region of Gabon and the Democratic Republic of the Congo, when gorillas or chimpanzees sick or dead from Ebola virus infection were consumed as "bush meat" (47). Studies by primatologists have found evidence of drastic reductions in the local populations of these animals, suggesting extensive virus spread (54). Some additional outbreaks in the Democratic Republic of the Congo during the period 2000–2010 appeared to be linked to bat exposures (55); samples from captured bats yielded Ebola RNA sequences but no infectious virus. The case fatality rates of outbreaks caused by *Zaire ebolavirus* during the period 1976–2013 frequently exceeded 70%.

The largest Ebola epidemic in history began in December 2013 when an infant in the village of Gueckedou, Guinea became ill, presumably following exposure to an infected bat. The virus spread to his family members, then was carried by chains of person-to-person transmission to multiple sites within Guinea. In March 2014, the outbreak was recognized by the government and by an MSF unit working in the country (4). After initial control efforts by local and foreign responders, the outbreak appeared to have been halted, but it was then discovered that infected individuals had traveled to Liberia and Sierra Leone, carrying the disease with them. By the summer, Ebola virus was spreading rapidly through the largest cities of the three countries abetted by the lack of medical and public health resources in one of the world's poorest regions. By fall, a massive international response helped create a large number of centers for the diagnosis and treatment of Ebola patients, staffed jointly by African and international medical workers (Figs. 6A, 6B).

A retrospective analysis found that the institution of treatment units has played a critical role in slowing the spread of the epidemic, preventing many thousands of cases (56). In addition to isolating patients and thereby reducing virus transmission, treatment units attempt to provide as much supportive care as possible. Although data on patient outcomes are limited, it appears that the overall case fatality rate for the epidemic is in the range of 30% to 40%, lower than what might have been expected based on previous experience in Central Africa (Table 1).

Several studies have examined the basic reproductive rate (R_0)—the number of persons to whom an infected individual is likely to transmit the disease—for Ebola Zaire virus. A review of epidemiological parameters noted that estimates of R_0 for past outbreaks had ranged from 1.4 to 4.7, while estimates for the West African epidemic had generally ranged from 1.2 to 2.5 (57). Traditional funerals have been a source of large numbers of infections, and the institution of safer practices is an important intervention to reduce transmission (58). The introduction of ETUs has both improved survival and reduced the spread of virus; in Liberia, R_0 decreased from 1.7 early in the epidemic to 0.1 after isolation and treatment became available (59).

FIGURE 6 The West African Ebola epidemic: patient care in a low-resource setting. A. Medical workers preparing to enter an Ebola treatment unit. A team approach ensures that PPE is donned and removed correctly. B. ELWA-3, the largest Ebola treatment center ever constructed, built by Médecins Sans Frontières adjacent to the ELWA mission hospital in Monrovia, Liberia. The center is designed to isolate persons possibly incubating Ebola virus disease, treat those who have become ill, and prevent the further spread of infection. (Médecins Sans Frontières, used with permission.)

Sudan ebolavirus

In the 1976 epidemic in Sudan, the first cases were seen among workers in a cloth factory in Nzara, which harbored large numbers of bats. Once the first patients were hospitalized, the virus spread to medical staff and family members (60). The largest Ebola Sudan epidemic occurred in Uganda in 2000, with more than 400 cases. An initial chain of transmission went unrecognized until student nurses in Gulu hospital began to develop a severe hemorrhagic illness. Because of the approximately 50% case fatality rate and the availability of several serum samples on many patients, retrospective analysis has identified a number of biomarkers predictive of survival or death (61–64).

FIGURE 7 The West African Ebola epidemic: patient care in a high-resource setting. Coauthor DC manipulating a peripherally inserted central catheter line of a critically ill Ebola patient requiring mechanical ventilation in the Special Clinical Studies Unit of the Clinical Center, National Institutes of Health, Bethesda, MD.

Côte d'Ivoire ebolavirus

In 1994, an ethologist studying chimpanzees in the Tai Forest of Côte d'Ivoire performed a necropsy on a recently dead animal, and 6 days later she became severely ill (65). The diagnosis of Ebola virus disease was made only after she had been evacuated to Switzerland; she survived, and no secondary transmission occurred. Despite its geographic origin, this virus is unrelated to the Zaire strain that caused the West African epidemic.

Bundibugyo ebolavirus

The fourth species of *ebolavirus* was identified in August 2007, when an outbreak of severe febrile disease occurred in western Uganda (66). The agent differed significantly from other Ebola species, but was distantly related to the Ivory Coast virus. The fatality rate of 25% among 149 confirmed cases was lower than that previously seen for the Zaire and Sudan viruses. Another outbreak occurred in eastern Democratic Republic of the Congo in late 2012, apparently originating from contact with bush meat; the case fatality rate was just over 50% (67).

Reston ebolavirus

The fifth Ebola species was recognized in 1989, when cynomolgus macaques imported from the Philippines to a quarantine facility in Reston, VA, developed a severe hemorrhagic illness, and electron microscopy examination of specimens unexpectedly revealed a filovirus (68). Several more outbreaks occurred over the following six years at other laboratories supplied by the source in the Philippines, but they stopped once the latter ceased operations. No illness was seen in workers who had been exposed to sick animals, but several were later found to have antibodies to the agent, suggesting that they had asymptomatic infection. The Reston agent is a distinct species of Ebola virus, which may have been exported from Africa at some time in the past. In 2009, the virus was unexpectedly detected in Philippine pigs (69); a local survey found antibodies in a small percentage of farmers with no history of severe illness. In contrast to its lack of virulence for humans, the Reston virus is highly pathogenic for laboratory primates.

Age-Specific Attack Rates

Prior to 2014, nearly all patients in outbreaks of filovirus disease in Africa were adults, probably because children are relatively unlikely to participate in the two activities by which individuals are most likely to become infected: hands-on patient care and preparation of a cadaver for burial. In contrast, infections in young children have been more common in the West African Ebola epidemic, possibly reflecting the very large number of infected persons and the occurrence of virus transmission within families. Recent analyses suggest that children differ from adults in their susceptibility to fatal Ebola virus disease. Patients under the age of 21 years have had lower case fatality than individuals over the age of 45 years (70, 71). However, children under 5 years of age are more likely to die than older children, with case-fatality rates approaching 90% (72).

Routes of Transmission and Risk Groups

Bats are known to have been the source of infection in a number of single cases and outbreaks of Marburg virus disease; the same or related species have presumably played a similar role in initiating some Ebola epidemics, but this remains unproven. Many people in West Africa have frequent contact with bats, providing opportunities for virus transfer (24). Exposure of wild apes to bat excretions presumably lies behind outbreaks that have resulted from their consumption as bush meat.

Once a human case has occurred through direct or indirect exposure to an infected animal, secondary infections follow when family members or medical workers caring for patients become contaminated with virus-containing body fluids. Nurses and doctors have played the tragic role of "sentinels" in several large hospital-based outbreaks, when patients with unrecognized filovirus disease were treated in the absence of infection control measures. The lack of such precautions can be explained in part by the fact that the most common severe febrile syndromes in Central Africa, such as malaria and yellow fever, are not transmitted by direct contact.

Transmission Routes

Filoviruses replicate principally in macrophages and dendritic cells, which are present in large numbers in the dermis and submucosal tissues; all body fluids will therefore contain virus. In addition, the body surface of a sick individual is often contaminated with virus-containing blood, vomitus, or feces, and replication in sweat glands and other dermal structures may deliver more virus to the skin. Infection may therefore readily be acquired by touching a patient, whether living or deceased, followed by transfer of virus from the hands to the mouth or eyes. During the West African epidemic, numerous episodes have been reported in which the ritual preparation of a cadaver for funeral services and subsequent touching of

the skin by mourners resulted in extensive transmission. These "super-spreading" events may have played a major role in the explosive expansion of the outbreak.

There is no evidence that filovirus disease spreads from person to person by any route other than direct contact with infectious body fluids. Although virus is present in the saliva and pulmonary secretions of patients, airborne transmission has not been proven in humans. Experimental infection via small particle aerosols (≤ 5 μm) has been demonstrated in the laboratory in rodents and nonhuman primates (73, 74). Secondary transmission of *Zaire ebolavirus* has been demonstrated between pigs and from pigs to nonhuman primates although these experiments did not distinguish between small versus larger (>5 μm) particle transmission (75, 76). Because blood and other body fluids can be aerosolized during medical procedures, all workers must use respiratory protection to avoid even a low risk of infection. The presence of virus in the breast milk of Ebola-infected mothers places infants at high risk of acquiring the disease (77).

Nosocomial Infection

The occurrence of large hospital-based epidemics of filovirus disease in Central Africa, beginning in 1976, has illustrated the potentially catastrophic consequences of bringing large numbers of sick people into close proximity in the absence of basic infection control regimens. Nosocomial infections were also common in the early phase of the West African epidemic, when doctors and nurses were either unaware that they were caring for Ebola patients, attempted to care for colleagues or family members outside of ETUs, or lacked the personal protective equipment and training in appropriate use to protect themselves against virus transfer. Fortunately, the incidence of nosocomial infection has decreased, as health care personnel in West Africa have been provided with personal protective equipment and training in their use. Accidental infection of three critical-care nurses has occurred during the treatment of Ebola patients evacuated to the United States and Spain; all survived their disease (78, 79).

Interestingly, the experience of nosocomial infections with filoviruses prior to the West African epidemic showed that the admission of a patient with unrecognized filovirus disease to a hospital with good hygienic practices typically resulted in few secondary infections. Thus, even though the doctors and nurses who cared for patients in the 1967 Marburg outbreak knew nothing about the novel disease or its mode of spread, only six of them became infected, and no tertiary transmission occurred (45). Similarly, the admission of a Marburg virus-infected traveler to a South African hospital in 1975 led to only a single case in an attending nurse (51). Two decades later, a Gabonese physician with unrecognized Ebola virus disease was treated without special precautions in a South African hospital, and only one member of the medical staff became infected (80). These histories suggest that basic measures, such as the use of gloves and hand washing, are the most important factors in preventing filovirus infection.

Readers should refer to publications on the websites of the US Centers for Disease Control and Prevention (http://www.cdc.gov/vhf/ebola/about.html) and the World Health Organization (http://www.who.int/csr/disease/ebola/en/) for current recommendations during travel, hospital care, and convalescence.

Duration of Infectiousness

In patients who survive filovirus disease, viremia tends to resolve by the end of the second week of illness, but some excretion may continue in the urine, and infectious virus may persist for weeks to months in "immunologically privileged" sites such as the seminiferous tubules of the testes, the anterior chamber of the eyes, and the central nervous system (see below). Following the 1967 Marburg outbreak, a patient who had recovered sufficiently to leave the hospital infected his wife, apparently through sexual intercourse (45).

The potential for transmission of Ebola virus by convalescent patients, months after their release from hospital, has caused concern that new cases may recur in regions free of the disease. After the 1995 Kikwit outbreak, some vaginal, rectal, and conjunctival swabs from one female patient were positive by reverse-transcription polymerase chain reaction (RT-PCR) at day 33, and virus was recovered from the semen of a male patient 82 days after disease onset (81). Similar observations have been made during the current West African epidemic. The semen of one male survivor in Sierra Leone was positive by RT-PCR 284 days after symptom onset (82). One instance of transmission of Ebola infection from a convalescent male to a sexual partner was proven by sequencing of virus from both individuals (83); in other cases, sexual transmission has been suspected but not proven by molecular testing (84). The ability of specific antiviral therapy to speed the clearance of infectious virus has not been investigated.

PATHOGENESIS IN HUMANS

The fulminant course and high case fatality rate of Ebola and Marburg virus diseases result from a combination of factors (85):

- The ability of filoviruses to suppress type I IFN responses and spread rapidly to macrophages, dendritic cells, and other cells throughout the body;
- The local and systemic effects of mediators released from infected cells, which may include acute gastrointestinal dysfunction;
- Fluid loss through vomiting and diarrhea, which may lead to hypovolemic shock;
- Extensive tissue damage caused by necrosis of infected cells;
- Impairment of adaptive immune responses, permitting uncontrolled viremia;
- Coagulation defects that accompany systemic inflammation and contribute to tissue ischemia.

Hemorrhage may occur, but bleeding is rarely the cause of death.

Patterns of Virus Replication

Filoviruses use macrophages and dendritic cells as their principal sites of replication (Fig. 8). Rapid viral replication and systemic spread are facilitated by two viral proteins that suppress type I interferon responses: VP35 inhibits IFN production by the infected cell, while VP24 blocks the response to exogenous IFN (11, 86). Infected macrophages migrate to local lymph nodes, while free virions released into the lymph and bloodstream disseminate to fixed macrophages in the liver, spleen, and other tissues, from which infection then spreads to hepatocytes, fibroblasts, and other cell types.

Because laboratory animal models of filovirus infection are characterized by uniform lethality, information on the relationship between levels of virus replication and outcomes can be obtained only from studies in filovirus outbreaks. A retrospective study of the outbreak of *Sudan ebolavirus* infection in Gulu, Uganda found that patients

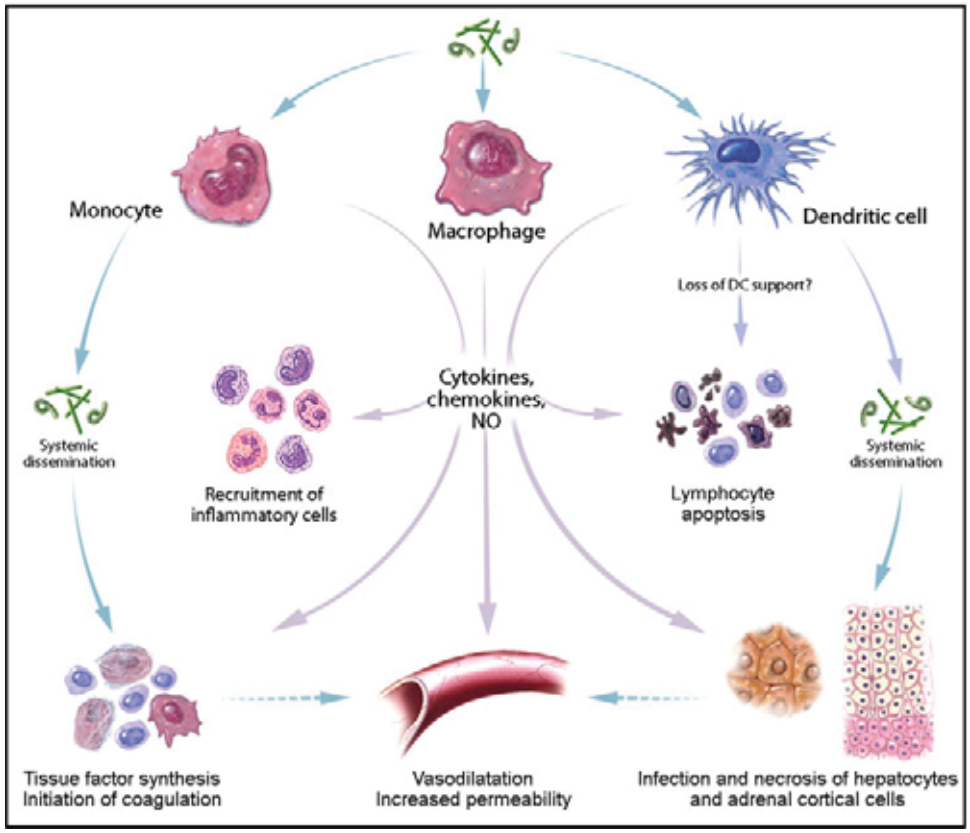

FIGURE 8 Pathogenesis of filovirus disease. Monocytes, macrophages, and dendritic cells are the primary sites of replication. Suppression of type I interferon responses permits rapid virus dissemination via the bloodstream to the spleen and other lymphoid tissues and to hepatocytes and parenchymal cells of other organs, resulting in further productive infection and multifocal necrosis. The release of proinflammatory mediators, the production of cell-surface tissue factor, and endothelial dysfunction contribute to coagulopathy, organ-specific vascular leak (e.g. lungs), and eventual multi-organ failure. Lymphocytes remain uninfected but undergo apoptosis, contributing to the failure of adaptive immune responses. Note: observations in patients during the West African Ebola outbreak suggest that damage to renal parenchymal cells and injury within the central nervous system plays a significant role in pathogenesis.

who died had circulating virus titers ("viral load") averaging 100-fold higher than those who survived (87). The positive correlation between the level of circulating virus and the likelihood of a fatal outcome has been confirmed in studies of cohorts of patients in the West African epidemic (70, 71). For example, a report from Sierra Leone based on 2,700 blood samples found that levels of viral RNA at the time of diagnosis were on average 100-fold lower in those who would survive their disease than in fatal cases (88). In another study, tracking of individual patients showed that survivors had significantly lower peak viremia levels than nonsurvivors and reached that level earlier (89). A decline from the peak level was observed in both groups, but it was more rapid in survivors. A third report stratified case fatality rates by the mean level of viremia during the first week of illness; for those with $<10e4.4$ copies/mL, the CFR was 21%, but it was 81% for those with $>10e5.2$ copies/mL (90). Because of the strong effect of circulating virus titer on disease outcome, future studies of novel therapies should be stratified by viremia level.

In addition to the widespread infection of macrophages and other cells and the systemic effects of circulating inflammatory mediators, the frequency of renal insufficiency and neurologic abnormalities in patients with Ebola virus disease suggests that these organs are also direct targets of viral infection. Filoviruses apparently do not replicate in lymphocytes, but massive numbers undergo apoptosis during the course of illness (91). In infected macaques, this consists of the early loss of natural killer (NK) cells, followed by $CD8^+$ and $CD4^+$ T cells; similar changes occur in mice (92).

The increased vascular permeability seen in filovirus disease was at one time attributed to direct infection and injury of the endothelial lining of blood vessels, but experimental studies in nonhuman primates have found no evidence of viral replication in these cells until late in the disease course and that the endothelium remains relatively intact even at terminal stages of disease (93). Retrospective assessment of cases of Ebola Sudan virus infection found that elevated serum levels of cytokines and chemokines, including IL-6, IL-8, IL-10, and MIP-1β, and of molecules associated with coagulopathy and altered endothelial function, including D-dimers, thrombomodulin, sICAM, and sVCAM, are associated with a fatal outcome (61, 64, 94, 95). Similarly, because there is little evidence of viral injury of the mucosal epithelium or submucosal tissues of the gastrointestinal tract (85), it appears that the vomiting and profuse diarrhea seen in many Ebola patients are also induced indirectly through viral or host-derived mediators.

Histopathological Features

Pathological changes in the tissues of filovirus-infected humans and animals can be divided into those caused directly by viral replication and those produced indirectly through host responses to infection (Fig. 8) (96). Among the former, the most prominent abnormality is the necrosis of virus-infected macrophages and dendritic cells in lymph nodes, thymus, spleen, liver, and other lymphoid organs, disrupting their normal architecture (Fig. 9A). Dissemination to parenchymal cells in the liver and other tissues also causes extensive injury. In the liver, virus spreads from Kupffer cells to hepatocytes, producing innumerable small foci of necrosis (Fig. 9B). Infected cells contain eosinophilic viral inclusion bodies, consisting of masses of viral nucleocapsids. Viral infection and necrosis of dermal cells and structures, including hair follicles and sweat glands, is apparently responsible for the extensive desquamation frequently seen in convalescent patients (97) (Fig. 10).

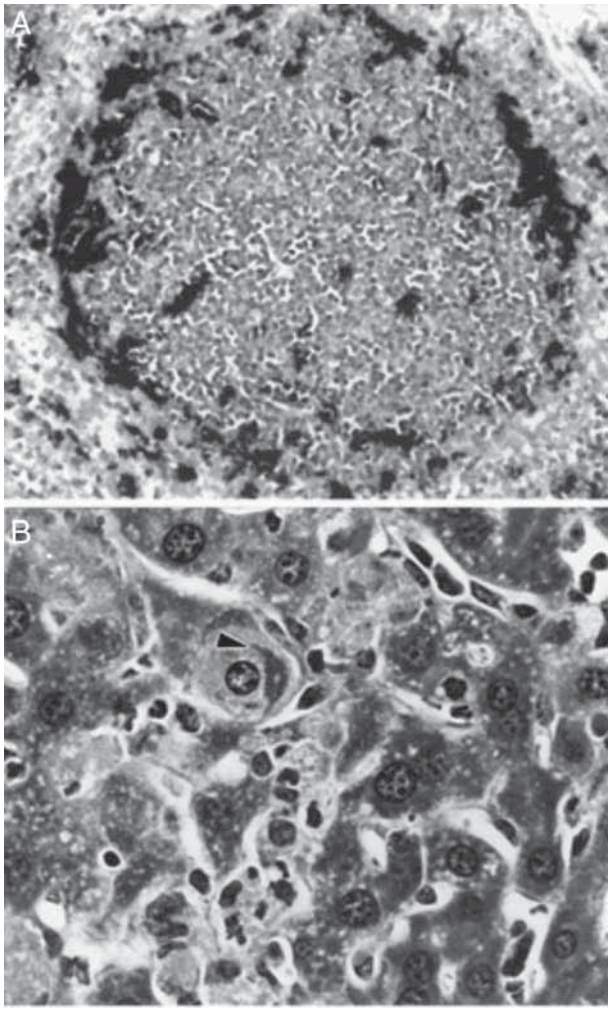

FIGURE 9 Replication of mouse-adapted Ebola virus in a lethally infected mouse. A. *In situ* hybridization of viral RNA in a splenic follicle; all marginal-zone macrophages contain replicating virus, while lymphocytes remain uninfected. B. Multifocal necrosis in the liver. Some hepatocytes contain acidophilic inclusion bodies (arrow), corresponding to the nucleocapsid aggregates shown in Figure 2B. (Courtesy of Tammy Gibb and Kelly Davis, USAMRIID.)

FIGURE 10 Extensive desquamation of the forearm and hand of a survivor of Ebola Sudan virus infection in Gulu, Uganda, 3 weeks after disease onset. (Courtesy of Dan Bausch, Tulane University.)

Histopathologic changes that result indirectly from filovirus infection can be separated into two categories. The first consists of manifestations of disseminated intravascular coagulation (DIC). Studies in laboratory primates have shown that the expression of cell-surface tissue factor causes infected macrophages to become encased in fibrin, most prominently in the spleen and other lymphoid tissues (98). Fibrin deposits are also seen in glomeruli and proximal tubules of the kidneys. The development of DIC also leads to perivascular and interstitial hemorrhage, most prominently in the bladder and in the lining of the gastrointestinal tract.

The second type of indirect injury in filovirus infection is the death of large numbers of uninfected lymphocytes in germinal centers of lymph nodes and lymphoid follicles of the spleen and thymus (91). Massive lymphocytolysis is a nonspecific accompaniment of septic shock and other types of severe infection, apparently induced by proapoptotic mediators and the disruption of normal physiological mechanisms that regulate lymphocyte populations (99). In combination with the destruction of dendritic cells and other antigen-presenting cells, lymphocyte apoptosis may prevent the generation of adaptive immune responses needed to eliminate viral infection.

Immune Responses

Nonspecific Responses: Cytokines and Other Proinflammatory Mediators

Cultured human macrophages infected with *Zaire ebolavirus* release the proinflammatory cytokines and chemokines tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), macrophage inflammatory protein 1*a* (MIP-1α), MIP-1ß, alpha interferon (IFN-α), and RANTES into the growth medium (100). The same mediators, plus IFN-ß, IFN-γ, and IL-18, and the potent vasodilator nitric oxide (NO) are present in the plasma of Ebola virus-infected nonhuman primates, and high levels of proinflammatory cytokines have also been detected in serum samples from acutely ill patients in African outbreaks (61, 101). NO levels were especially high in fatal cases in the 2000 Ebola Sudan outbreak (61).

The expression of cell-surface tissue factor by infected macrophages also plays a critical role in producing DIC by triggering the extrinsic coagulation pathway (98). Because tissue factor production begins with the first infected

macrophage, coagulopathy can be detected early in the disease course. In macaques, D-dimers are the first disease marker detected in the plasma, and they are extremely elevated in the plasma of patients dying from Ebola virus disease (94, 98).

Specific Immune Responses

Most fatally infected Ebola patients have persistent high-level viremia, usually without a detectable antibody response. In contrast, patients who survive the disease develop virus-specific IgM and IgG responses, generally during the second week of illness (41, 87). These antibodies are evidently long-lasting, because a retrospective study of survivors of the 2000 Ebola Sudan epidemic observed neutralizing antibodies in some individuals up to 10 years after infection (102). Cell-mediated immunity also plays an important role in survival: sequential blood samples from four accidentally infected medical workers who were successfully treated at Emory University Hospital showed intense activation of both Ebola virus specific and nonspecific B and T cells, with large numbers of circulating plasmablasts and high percentages of activated CD4+ and CD8+ T cells, accompanied by production of virus-specific antibodies (103). These observations are consistent with our current understanding of pathogenesis, in which Ebola survivors are those who manage to mobilize an early adaptive immune response, while fatal cases fail to do so, at least in part from the destruction of infected dendritic cells and the loss of lymphocytes through apoptosis.

Correlates of Disease Resolution

Efforts to identify features of filovirus disease and host responses that are predictive of survival have greatly expanded with the epidemic in West Africa. The principal finding has been that the ability of the patient's immune system to control viral replication is the fundamental determinant of survival: those who develop a virus-specific antibody response, typically in the second week of illness, will control and eliminate the agent, while patients lacking such a response will have continuous, high-level viremia until death (41, 61). Young patients who have a relatively low viral load at the time of presentation to a medical facility are most likely to survive; the development of severe diarrhea and markedly elevated serum creatinine values, indicative of renal failure, are principal predictors of a fatal outcome (70).

The roughly 50% survival rate in outbreak of Ebola Sudan virus infection in Gulu, Uganda made it possible to examine whether human genetic variation might influence the outcome of infection (61). Sequence-based HLA-B typing of isolated leukocytes showed that alleles B*67 and B*15 were linked to a fatal outcome, while B*07 and B*14 were more common in survivors. Such research has not yet been reported from the epidemic in West Africa, but the large number of patient samples and data on the course and outcome of illness should make such investigation possible.

CLINICAL MANIFESTATIONS

Prior to 2014, detailed descriptions of filovirus disease could only be found in clinical reports from the 1967 Marburg outbreak, a few patients hospitalized in South Africa and a handful of accidental laboratory infections. In contrast, the West African epidemic has been accompanied by a large number of articles describing the clinical features of patients. Our comparison of early and recent reports has led us to the following conclusions:

- Marburg and the various species of Ebola virus cause similar clinical syndromes, differing principally in the case fatality rate.
- The disease that has occurred since late 2013 in West Africa resembles that observed in prior outbreaks in Central Africa.

As noted previously, Marburg and Ebola virus infections were classified by those who first studied them as types of "viral hemorrhagic fever," employing terminology introduced in the 1930s. Once filovirus disease had received that label, researchers studying laboratory animals and physicians caring for patients tended to focus on the development of coagulation defects and bleeding, even while recording that fatal hemorrhage was relatively rare. Other aspects of the filovirus syndrome, including the vomiting and profuse diarrhea seen in many patients in West Africa, were noted in clinical reports, but given relatively little attention.

Typical features of Ebola virus disease and associated abnormalities in laboratory tests are listed in Table 2. In contrast to earlier descriptions of "Ebola hemorrhagic fever," in which shock was attributed to diffuse vascular leak, clinicians now recognize that severe fluid loss from vomiting and diarrhea early in the course of illness may lead rapidly to shock, while the hemorrhage that was once assumed to be a major cause of death is relatively rare.

Incubation Period

The incubation period of Ebola virus disease averages 6 to 12 days (range 2 to 21 days), followed by the onset of fever, generalized malaise, fatigue, and body aches. Although "asymptomatic" infections in persons in close contact with Ebola patients were described in a report from Gabon in 2000 (43), such cases have not been reported since. Findings described by organ system are noted below.

Gastrointestinal

Large-volume watery diarrhea is a common feature of Ebola virus disease, often accompanied by episodes of severe nausea and vomiting, which may result in significant intravascular volume depletion (70, 104, 105). Diarrhea begins within 5 days of fever onset, and 5 or more liters of stool per day may be produced. Watery consistency of diarrhea and a large stool volume suggest a small-bowel, and possibly a whole-bowel, secretory process (106). Mild inflammation has been observed in the lamina propria of fatal cases, suggesting an inflammatory component, although confirmatory clinical studies evaluating for fecal leukocytes or lactoferrin have not been reported (85).

Oral ulcers, dysphagia, and odynophagia may contribute to decreased oral intake. Severe upper and/or lower gastrointestinal bleeding occurs in about 5% of patients, typically just before death. Reports of elevated serum amylase levels in the setting of abdominal pain suggest that pancreatitis may also complicate the disease (107).

Neurologic

Patients with Ebola virus disease frequently develop profound neuromuscular weakness and pain, manifested by loss of energy and strength, beginning before or shortly after fever onset (108). Weakness develops acutely, is typically symmetrical, and is often progressive, involving both upper and lower extremities. Postural instability contributes to difficulty walking, and in severe cases respiratory muscle dysfunction results in ineffective ventilation. Myositis contributes to muscle pain and weakness with creatinine phosphokinase

TABLE 2 Clinical and laboratory manifestations of Ebola virus disease

Stage of illness	Time post-onset	Clinical	Laboratory
Early febrile	Days 1–3	Fever, malaise, fatigue, body aches	Decreased WBC, lymphocyte and platelet counts Elevated AST, ALT, hemoglobin, and hematocrit
Gastro-intestinal	Days 3–10	Primary: Epigastic and abdominal pain, nausea, vomiting, diarrhea	Leukocytosis (increased neutrophils and band cells)
		Associated: Persistent fever, asthenia, conjunctival injection, chest pain, dysphagia, odynophagia, arthralgia, myalgia, hiccups, delirium, rash	Persistently elevated AST/ALT, thrombocytopenia Increased BUN, creatinine,hypo or hyper kalemia, hypomagnesemia Hyponatremia, albuminemia Elevated PT/PTT/INR and fibrin split products
Shock, organ failure, and other complications	Days 7–12	Renal and/or respiratory failure (oliguria, anuria, tachypnea) Encephalopathy and/ or meningoencephalitis, (diminished consciousness, agitation, stiff neck, seizures) Secondary bacterial infection GI hemorrhage	Findings may overlap with prior stages of illness. In addition to previous findings: Elevated lactate Decreased bicarbonate, Hb, HCT Hypoxemia
Recovery	Days 7–12	Resolution of abnormal signs and symptoms, increased oral intake, energy	Resolution of laboratory abnormalities
Convalescence	Up to six months	Arthralgia, myalgia, fatigue, abdominal pain, uveitis, hearing loss, neuro-cognitive abnormalities	

Abbreviations: WBC: white blood cell; AST: aspartate aminotransferase; ALT: alanine aminotransferase; BUN: blood urea nitrogen; PT: prothrombin time; PTT: partial thromboplastin time; INR: international normalizing ratio; Hb: hemoglobin; HCT: hematocrit.

elevations observed between 300 and 3,500 international units/L (71). More significant elevations, consistent with rhabdomyolysis, typically in the 10,000 to 25,000 IU/L range (109), have not been reported, suggesting that profound weakness cannot be attributed to myositis alone.

Delirium occurs during the peak of illness and may manifest as disorientation with difficulty arousing, confusion, or combativeness. Encephalopathy may be attributable to viral infection of the CNS, secondary host responses, or metabolic derangements, including high or low serum sodium levels, hypoglycemia, or uremia. Panencephalitis has been documented in Ebola-infected nonhuman primates (110) and in two fatal human cases of Marburg virus disease (85). Meningoencephalitis is manifested by headache, neck rigidity, delirium, and diminished consciousness typically during acute illness or early after initial recovery (105, 111, 112).

Seizures occur in approximately 5% of cases, typically as preterminal events (70). Intractable vomiting and occasionally hiccups are also observed. Despite the development of profound acute neurologic abnormalities, patients with EVD who receive adequate supportive care may achieve near-complete recovery (113).

Ocular

Individuals may manifest signs and symptoms of uveitis (e.g., blurred vision, photophobia, blindness) during the acute illness (114). However, the etiology of these findings remains unclear, because detailed ophthalmologic examination including fundoscopy, have not been reported.

Cardiac

Ebola virus can infect the endocardium, and clinical pericarditis and myocardial dysfunction have been reported (115, 116). However, the contribution of viral infection to structural or functional cardiac abnormalities has not been well characterized. Heart-rate responses in patients are frequently lower than would be expected in the setting of high fever and dehydration (107), which may result in an underestimation of intravascular volume depletion and diminished cardiac compensatory ability in the setting of shock. Peripheral edema may be observed in the setting of aggressive fluid resuscitation and low serum albumin levels, following renal failure, or in the setting of secondary bacterial sepsis resulting in vasodilatory shock.

Sudden death of uncertain etiology has occurred in West African Ebola patients who appeared to be recovering (105). Some of these deaths may be the result of low serum magnesium or potassium levels, which in combination with haloperidol, quinolone antibiotics, ondansetron, lumefantrine for malaria prophylaxis, and other drugs that prolong the QT interval, may predispose to the arrhythmia known as "torsade de pointes."

Respiratory

Probably because filoviruses do not induce upper respiratory tract symptoms and thorough assessment of the lungs is often not possible in low-resource settings, respiratory complications have largely gone unrecognized. However, the viruses are known to infect alveolar macrophages and pulmonary endothelial and interstitial cells (85). Lower respiratory tract complications, including multifactorial respiratory failure, have been observed in patients treated in high-resource settings (79, 116, 117).

Pulmonary injury results in noncardiogenic interstitial pulmonary edema and pleural effusions, which may be made worse by aggressive fluid resuscitation (79). Neuromuscular weakness predisposes to atelectasis, aspiration, and secondary bacterial pneumonia, as well as ineffective or dead-space ventilation, manifested by rapid, shallow breathing.

Multifactorial hypoxia and ineffective ventilation contribute to the development of respiratory failure (Fig. 7).

Hepatic

Moderate to severe hepatitis typically begins early in the disease course, worsens progressively, and then improves with declining viral burden. Serum aspartate aminotransferase (AST) levels becomes elevated more than alanine aminotransferase (ALT) levels, typically in a 3:1 or greater ratio, suggesting some AST elevation may derive from sources other than liver (e.g., muscle) (70, 107). Multifocal necrosis with minimal inflammation is observed pathologically, with infection of hepatocytes, sinusoidal endothelial cells, and resident Kupffer cells (85). However, fulminant hepatitis with profound elevation in transaminases has not been reported. Elevated serum bilirubin may occur in the absence of jaundice, probably resulting from intrahepatic cholestasis following hepatocellular injury.

Hypoglycemia, hypoalbuminemia, and clotting abnormalities are observed in severe disease. This suggests hepatic synthetic dysfunction, but severe hypoglycemia is more likely the result of acute depletion of liver and muscle glycogen and increased metabolic demand in the setting of critical illness. Rather than being the result of diminished hepatic production, albumin may be lost in stool and urine, and clotting defects are more likely attributable to consumption of coagulation factors.

Genitourinary

Renal insufficiency is common in Ebola virus disease, leading to renal failure in severe and fatal cases. The etiology of acute kidney injury is likely multifactorial. Autopsies have shown that the virus infects renal tubular and glomerular endothelial cells, with evidence of acute tubular necrosis (85), which might be induced through direct cytotoxic injury, hypoperfusion due to shock, and/or microvascular abnormalities. Another contributing factor might be myoglobin-induced renal tubular injury due to muscle breakdown.

Hematologic

Leukopenia develops early, probably related to the indirect killing and depletion of lymphocytes in the spleen, lymph nodes, and other lymphoid tissues. White blood cell counts then recover, resulting in a neutrophil-predominant leukocytosis. Platelet counts decline early in the disease, with nadirs in the range of 30,000/mm^3. Bone marrow evaluation in fatal cases has shown normal megakaryocyte numbers, suggesting that thrombocytopenia results from consumption rather than diminished production (85). Reactive thrombocytosis may be observed during recovery (78).

Increases in prothrombin time, partial thromboplastin time, international normalizing ratio, and fibrin split products, with low fibrinogen levels, also suggest the presence of consumptive coagulopathy in severe cases. Elevated hemoglobin and hematocrit may be observed early in the course of illness, suggesting hemoconcentration. Anemia typically follows, perhaps attributable to hemodilution in the setting of fluid resuscitation, gastrointestinal blood loss or increased peripheral destruction due to hemolysis.

Endocrine

Based on studies in laboratory animals, it has been postulated that adrenal infection and necrosis may play an important role in pathogenesis (118). Patients show nonspecific signs and symptoms observed during adrenal insufficiency (e.g., lethargy, fever, vomiting, hypoglycemia). However, physiology of shock in EVD appears most consistent with hypovolemia and not vasodilatory (distributive) shock that is classically observed in adrenal crisis. Distributive shock has been described in patients with secondary Gram-negative bacterial sepsis (111), and adrenal insufficiency should be considered in refractory shock (117).

Skin

Patients with Ebola or Marburg virus disease often develop an erythematous, nonpruritic maculopapular rash on the trunk and extremities during the first week of illness; it is typically more apparent in light-skinned individuals. The rash typically becomes confluent and may desquamate (Fig. 10) (107). In the setting of thrombocytopenia, a self-limited petechial rash may also be observed in areas where pressure is applied to the soft tissues. Patients are predisposed to skin breakdown and pressure necrosis because of immobilization, poor nutrition, and low serum albumin levels.

Concurrent Infections

Patients with filovirus disease may develop bacterial coinfections, leading to bacterial sepsis (111). Infrequent access to microbiologic testing has limited knowledge of the actual frequency of concurrent infections in outbreak settings and their contribution to overall mortality (119). The degree to which malaria, tuberculosis, HIV, and other chronic infections common in West Africa have added to morbidity and mortality during the current epidemic is also not known.

Pregnancy

EVD during pregnancy is associated with high risk of fetal and maternal death, typically associated with severe hemorrhage (120). Atypical presentation may occur in pregnancy because fatal EVD has been reported in the absence of either reported or detected fever (121). No data are available to suggest a benefit of caesarean versus vaginal delivery, although survival of the mother following vaginal delivery has been reported (122). Viral RNA has been detected in placenta and fetal blood up to 7 days after clearance from maternal blood (122).

Strict infection control is required in the care of pregnant women with potential EVD exposures and for those in labor even after EVD recovery. Live virus has been detected in breast milk of infected mothers up to 16 days after EVD onset (38). Consequently, breastfeeding of susceptible infants should be avoided until risk of transmission can be ruled out.

Late Complications and Sequelae

Long-term sequelae include blurred vision, retroorbital pain, hearing loss, neurocognitive deficits, sleep difficulties, fatigue, and arthralgias (123). Multiple studies of survivor cohorts for these and other medical problems are in progress.

Well-documented late complications of EVD, attributable to the persistence of virus in immune protected sites, include uveitis and meningoencephalitis. Ophthalmic complications in survivors of Ebola virus disease were first described after the 1995 Kikwit outbreak (114). One month or more into convalescence, patients developed eye pain and loss of visual acuity, and physical examination revealed uveitis. Similar cases have occurred in the current West African epidemic, and testing has found live virus in samples of aqueous humor months after clearance from the bloodstream (124). One case of late-onset meningoencephalitis beginning 10 months after initial clinical recovery has recently been described in which virus RNA was detected in CSF and blood; full recovery was seen after experimental

antiviral therapy (see below) (125). As discussed above, the persistence of virus in the semen of an Ebola survivor for up to six months resulted in sexual transmission (83).

LABORATORY DIAGNOSIS

Diagnostic tests for filovirus infection are based on the detection of viral RNA or antigens in patient samples, or occasionally of a virus-specific antibody response (126). Because all symptomatic patients are viremic, serum is the best specimen for testing. The need to collect and process many thousands of blood samples during the current Ebola epidemic in West Africa has resulted in the creation of guidelines for diagnostic testing by the U.S. Centers for Disease Control and Prevention (http://www.cdc.gov/vhf/ebola/diagnosis/index.html).

Virus Isolation

Filovirus isolation through growth in cell culture has long been considered the "gold standard" for diagnosis, but it is performed more often for research than diagnostic purposes. Any deliberate propagation of virus must be performed in a BSL-4 containment laboratory under adequate biosurety conditions. Vero cells (usually the E6 clone) are most widely used to culture virus from clinical samples.

Antigen Detection

Before the development of nucleic acid-based methods, filovirus detection in field settings was often based on detection of viral antigens by antigen-capture ELISA because it could be performed without a source of electricity (41). In response to the need for rapid point-of-care diagnosis in the West African epidemic, a novel antigen-based assay has been developed that employs a finger-stick blood sample and gives a result in 15 minutes (127). In contrast to RT-PCR, which requires electrical power and skilled technicians, the ReEBOV™ Antigen Rapid Test (Corgenix Medical Corp.) can be performed in the field by local health workers. A blinded comparison of the assay with a standard RT-PCR method found that the point-of-care test correctly identified 92% of patients and 85% of uninfected controls. Information on additional antigen-based diagnostic assays is available on the WHO website (128). A diagnosis of Ebola or Marburg hemorrhagic fever can also be made postmortem through immunohistopathological study of a formalin-fixed skin biopsy sample (97).

Nucleic Acid Detection

The most sensitive diagnostic assay, which has been performed many thousands of times during the current Ebola outbreak, employs RT-PCR, usually in the modified form of real-time (quantitative) RT-PCR, which can provide a diagnosis and measurement of viral load in an inactivated patient specimen within two hours. Real-time RT-PCR is being employed both to confirm Ebola virus disease before admitting a patient to a treatment center and to discharge survivors from a center, after two negative tests have been obtained over at least 48 hours. The use of sequence-based assays has been facilitated by the close genetic relationship of the West African virus to earlier Zaire isolates and the origin of all chains of transmission from a single source.

RT-PCR was first used to diagnose Ebola virus disease in 2000 in Gabon (129), and real-time RT-PCR was introduced two years later (130). The new methods were more sensitive than antigen-based tests for rapid diagnosis. In the Gulu epidemic, RT-PCR identified infected patients 24 to 48 hours earlier than an ELISA method (61). As discussed elsewhere, the quantitation of circulating virus has prognostic significance because fatally infected patients have persistently higher circulating viral titers, which remain elevated through death. It will likely also prove important in therapeutic monitoring with respect to antiviral interventions.

Serologic Assays

The detection of filovirus-specific antibodies in serum has been used both to diagnose patients during outbreaks and to assess the prevalence of exposure to filoviruses among various population groups. In patients with Ebola or Marburg virus disease, the development of a virus-specific antibody response is an important prognostic marker because those who fail to produce an antibody response by the second week of illness are unlikely to survive (41). IgM disappears from the serum during convalescence, but specific IgG persists for at least several years.

Serosurveys carried out in Central Africa during the 1980s made use of immunofluorescence assays, which had a high background level of positivity, suggesting a high prevalence of filovirus infection in regions where the disease had never been observed. The method has since been abandoned in favor of IgG ELISA for population screening, but even this more specific test has given a positive signal in samples from persons outside of Africa. For the results of a serosurvey to be meaningful, the specificity of the assay must be proven by including numerous control samples from people outside the region of interest.

PREVENTION

General

In Africa, primary filovirus infections of humans occur only as a result of direct or indirect contact with chronically infected bats or with sick or dead nonhuman primates. Prevention efforts in endemic areas focus on preventing such contact. Access to some abandoned mines and caves in Uganda and the Democratic Republic of the Congo inhabited by infected bats has been blocked and residents warned of the danger of bat exposure. In regions of Central Africa where Ebola virus disease is endemic, educational efforts have been initiated to warn local residents of the danger posed by gorillas, chimpanzees, and other animals found sick or dead in the forest.

Active Immunization

Studies in nonhuman primates have shown that exposure to the antigens of the two filoviral genera does not induce cross-protective immune responses: vaccines that protect macaques against Marburg virus are inactive against the Ebola viruses and vice-versa. Significant antigenic differences also exist among the various Ebola virus species. Because the Zaire and Sudan viruses have caused nearly all outbreaks, experimental vaccine formulations have contained antigens of one or both species.

Successful immunization of nonhuman primates against Ebola virus was first demonstrated in 2000, when macaques that had received three doses of a DNA vaccine encoding the *Zaire ebolavirus* surface GP, followed by one dose of a recombinant replication-deficient human adenovirus-5 encoding the same antigen, were protected against an otherwise lethal virus challenge (131). It was subsequently found that the Ad-5 vaccine given alone was sufficient to induce rapid protection (132). A few years later, recombinant vesicular stomatitis virus (VSV) vaccines encoding the

Marburg or Ebola virus GP were also shown to induce rapid protection in macaques (133–135). A number of other vaccine platforms have also been shown to protect nonhuman primates against Marburg or Ebola virus, but all of them have required more than one vaccine dose to elicit solid immunity. The current status of Ebola vaccine platforms can be determined from the latest update of the WHO report of research and development of drugs, vaccines, and diagnostics (136). However, because a fast-acting vaccine was clearly needed in response to the expanding Ebola epidemic in West Africa, the adenovirus- and VSV-vectored vaccines were given priority for development. The following discussion therefore focuses on these two approaches.

Few humans have been naturally exposed to VSV but adenovirus infections are common, and researchers have therefore been concerned that preexisting immunity to the vector might reduce the immunogenicity of a recombinant adenovirus vaccine. This proved to be the case in the first Phase I trial of an Ad5 Ebola vaccine, which detected significantly lower antibody responses to the Ebola Zaire GP in vaccinees whose serum contained antibodies to Ad5 (137). Because antibodies to Ad5 are common in all human populations and almost universal in Africa (138), developers evaluated two other human serotypes, Ad26 and Ad35, to which human populations show much lower antibody prevalence, as vectors for Ebola GP expression. However, an initial evaluation of an Ad26-vectored Ebola Zaire vaccine in macaques observed only partial protection in animals that had received a single vaccination, although full protection was achieved by boosting with the same antigen encoded by Ad35 (139).

In the meantime, other workers had found that adenoviruses isolated from chimpanzees were highly immunogenic (140); a chimpanzee adenovirus, ChAd3, as a vector for a *Zaire ebolavirus* vaccine provided rapid, uniform protection in macaques after a single immunization (141). Resistance to challenge waned after several months, but solid immunity was reestablished by boosting with a quadrivalent modified vaccinia Ankara (MVA) vector encoding the GP of the Zaire, Sudan, and Tai Forest Ebola species and of Marburg virus. No safety concerns were identified in dose-ranging Phase 1 studies in the United States and in West Africa (142); a dose of 10^{11} viral particles elicited antibody responses expected to be protective in a field setting. Boosting with the tetravalent MVA vaccine was also well tolerated. Although such a prime-boost strategy could not be used to elicit rapid protection during an outbreak, it could provide durable, broad-spectrum immunity in laboratory researchers and medical workers who might respond to future epidemics.

As noted above, recombinant vesicular stomatitis virus (rVSV) vaccines encoding the Marburg or Ebola virus GP induce rapid protection in rodent and primates models (133–135). They are also able to prevent mortality or prolong the time to death of macaques when administered one week before virus challenge or soon after (< 1 hour for Ebola [143] but up to 48 hours for Marburg [144]), properties that are highly desirable in the setting of an epidemic. However, in contrast to vaccines based on replication-defective adenoviruses, rVSV vaccines employ a live, replication-competent virus that circulates in the bloodstream during the first few days postvaccination, potentially inducing illness and fever resembling the onset of filovirus infection. Phase I trials of a recombinant VSV Ebola vaccine observed transient viremia in most subjects and fever in about one third; at one study site, 22% of subjects developed transient arthritis in the second week, and some also developed localized skin vesicles; the vaccine virus was identified in vesicular fluid of two vaccinees and synovial fluid of one (145, 146). Disabling but self-limited systemic illness, fever, and viremia were also observed in a physician given a 5-fold higher dose of the rVSV vaccine as post-exposure prophylaxis (147).

Following a careful risk-benefit assessment, the rVSV Ebola vaccine was advanced to a controlled evaluation of efficacy in Guinea. Investigators employed a novel cluster-randomized "ring vaccination" strategy, in which the diagnosis of a new case of Ebola virus disease was followed by the immediate identification of the patient's contacts and their contacts; adults within each such "cluster" were then randomized either to immediate rVSV vaccination or vaccination after 21 days (148). No cases of Ebola virus disease with onset 10 or more days after randomization were seen in the immediately vaccinated contacts compared to 16 cases in those who received delayed vaccination (direct vaccine efficacy 100%; 95% CI, 74.7%-100%), and there was some evidence for an indirect benefit for nonimmunized persons within clusters. This success led to early termination of the trial, and the vaccine is now being administered to all patient contacts.

The adenovirus and rVSV vaccine platforms differ in the mechanism of induced immunity. A study in nonhuman primates found that resistance to Ebola virus elicited by a recombinant adenovirus vaccine was based primarily on CD8+ T cells, and an antibody response was not required (149). Macaques immunized with the rVSV Ebola vaccine do not develop significant levels of neutralizing antibodies; instead, protection correlates with the titer of antigen-binding antibodies, as measured by ELISA (150). Its efficacy also does not require a cell-mediated immune response, as macaques depleted of CD8+ T cells were still protected (151).

Immunogenicity, including durability of responses, and safety data are being generated from other studies of ChAd3-, rVSV-, Ad26-, and MVA-vectored Ebola virus vaccines. MVA boosting of the ChAd3 vaccine provides a marked increase in antibody titers and is expected to significantly lengthen protective immunity (152). An NIH-sponsored large-scale, placebo-controlled trial of the ChAd3- and VSV-vectored vaccines ("PREVAIL") began in Liberia in early 2015 (153). An unblinded, randomized trial of the VSV Ebola vaccine ("STRIVE") was also initiated in Sierra Leone (154).

Postexposure Prophylaxis

Antiviral drugs or antibodies that have shown therapeutic efficacy against filoviruses in laboratory animals might be used to prevent illness in persons who have suffered high-risk virus exposures. A UK report described four medical workers in the West African epidemic who had needle-stick injuries and were treated with the novel antiviral favipiravir, with or without a monoclonal antibody preparation; none became ill (155). Immediate postexposure treatment with favipiravir has been advocated in field settings but not subjected to rigorous testing (156).

Immunization with a fast-acting vaccine is also a potential means of postexposure prophylaxis. As noted above, an rVSV vaccine encoding the Marburg surface glycoprotein protected macaques when inoculated 30 minutes after an injection of Marburg virus and provided partial protection as late as 48 hours after an otherwise uniformly lethal virus exposure, while rVSV vaccines against Ebola viruses prevented death of 50% of animals when administered 20 to 30 minutes after Zaire or

Sudan virus challenge (135, 143, 145, 146, 157). The rVSV-*Zaire ebolavirus* vaccine has been given to several accidentally exposed medical workers in the West African epidemic, In one case, a physician was vaccinated 43 hours after an accidental needlestick and developed fever and malaise 12 hours later; testing of blood samples revealed circulating vaccine virus but no Ebola virus infection (147). In macaques, postexposure vaccination does not provide cross-protection between Marburg and Ebola viruses, indicating that it is not based on the induction of innate immunity but on the rapid development of antigen-specific responses.

Management of Outbreaks

Because filoviruses spread from person to person only through direct physical contact with virus-containing body fluids, any outbreak can in principle be halted by traditional public health measures. These include finding, isolating, and treating all persons with a history of possible virus exposure; finding and monitoring everyone who has been in close contact with those persons; and continuing rigorous surveillance until no new cases have occurred during a time equal to twice the incubation period of the disease. In practice, as the epidemic in West Africa has demonstrated, ending a filovirus outbreak may be much more difficult. Simply establishing a diagnostic capability and constructing isolation and treatment units will be insufficient if members of the local population fail to understand the nature of the disease, distrust local and foreign medical workers, and see entry into a treatment center as a death sentence. Massive public education efforts may be needed to convince the public that cooperation with health authorities provides the best opportunity for survival.

Before an outbreak can be controlled, it must be recognized, so the initial challenge is for medical workers to suspect or diagnose the first cases of filovirus disease against the background of febrile illnesses common in Africa. Unfortunately, doctors and nurses have often acted as "sentinels" when caring for patients with unrecognized Ebola or Marburg virus infection, assuming that a patient has malaria or another condition that is not transferred through physical contact. Once a filovirus is known to be present and a response is under way, the establishment of a diagnostic laboratory and the testing of blood samples from sick individuals with an appropriate contact history can identify those who should be isolated.

Perhaps the most difficult aspect of controlling a filovirus outbreak is the establishment of trust between medical responders and those they are trying to help. The arrival in a village of aid workers clad from head to foot in protective gear and their attempts to convince patients and their contacts to enter a frightening isolation ward can cause sick persons to hide or flee and the general populace to resist the team's efforts. Fear may be further heightened by rumors that the strangers are actually spreading the disease or are using it to conduct experiments on Africans. Such a reaction may be unavoidable as long as a diagnosis of filovirus infection is equivalent to a death sentence. To counteract it, medical workers must convince the local population that treatment in an isolation unit provides the best chance of survival; their success in doing so may be largely responsible for control of the West African epidemic.

TREATMENT

Supportive Care

The level of supportive care that can be provided to patients with Ebola virus disease influences survival and depends on the setting and the available resources (Table 3). During the current Ebola epidemic, the vast majority of cases in West Africa have been managed in variably equipped field treatment centers, while a few have been transported to hospitals in Europe and the United States with advanced critical care capacity. In past outbreaks, case-fatality rates approached 90% in the absence of supportive care. In contrast, mortality in field centers in West Africa has generally ranged from 40% to 60% and have been 20% or less for patients treated in modern hospitals. The differences in overall mortality observed in these settings suggest that survival correlates with the availability of adequate medical staff, supplies, and equipment.

A basic factor in the quality of care is the frequency and duration of interactions between health care workers and their patients. In low-resource settings such as West Africa, the high ratio of Ebola patients to providers, combined with extreme heat exposure due to the tropical environment and restrictive personal protective equipment, severely limit bedside care. The resulting inability to control symptoms, limit, or replace fluid and electrolyte losses and address complications in a timely fashion predisposes to fatal outcomes. In contrast, the continuous bedside monitoring available in a high-resource setting permits improved recognition and management of clinical manifestations and complications. Peripherally inserted central catheters or standard central venous catheters allow for frequent laboratory testing and reliable administration of intravenous therapies (Fig. 7).

Management in the early phase of illness includes relief of fever, pain, and other symptoms. Paracetamol and acetaminophen have been used safely but may require dose adjustment in the setting of hepatic dysfunction. Nonsteroidal antiinflammatory drugs are not recommended, given the frequency of renal insufficiency. Salicylates are relatively contraindicated. Narcotics such as morphine may be used for severe pain, but diminished or altered mental status, contributing to delirium and inability for self-care, may limit dosage.

As noted above, agitated delirium is common in Ebola patients, predisposing them and their providers to harm. Antipsychotic medications such as haloperidol are effective but are associated with QT prolongation and should be used with caution. Benzodiazepines also reduce agitation but typically produce sedation that limits the patient's ability for self-care, predisposing to dehydration, aspiration pneumonia, and other complications. In high-resource settings, close monitoring of neurologic function allows early recognition of complications, including encephalopathy and seizures. Intubation with continuous sedation and analgesia can be safely accomplished, if needed.

In the absence of supportive care the recurrent vomiting and large-volume watery diarrhea that typically develop within 5 days of fever onset may lead to hypovolemic shock and death (70, 104, 105). Even with close attention to fluid management, sequential organ dysfunction or failure may occur, necessitating advanced supportive care, including mechanical ventilation and renal replacement therapy, if available. Fluid losses may be limited with the use of antiemetic and antidiarrheal medications. Vomiting was effectively reduced in Ebola patients given metoclopramide (106), which carries a low risk of QT-interval prolongation, but dosage adjustment in renal insufficiency is recommended. Ondansetron has also been used, but there is a risk of QT prolongation. Promethazine is not recommended, given neurologic and cardiovascular risks.

TABLE 3 Recommendations for management of filovirus disease in low- and high-resource settings

Clinical feature	Management recommendations	
	Low resource	High resource
General	Provide nursing care as the situation permits	Continuous bedside nursing with close clinical monitoring and management
	Place peripheral IV line for initial blood draw and IV therapy	Place PICC or central venous catheter for frequent blood draws and IV therapy
	Treat fever and moderate pain with paracetamol or acetaminophen	Treat fever and moderate pain with paracetamol or acetaminophen
Neurological	Administer benzodiazepines (e.g., valium) for agitation or seizures	Continuously sedate intubated patients (e.g., midazolam, dexmedetomidine)
	Administer narcotics (e.g., morphine) for severe pain	Administer narcotic drip (e.g., fentanyl) for pain in intubated patients
	Avoid over-sedation with benzodiazepines and narcotics	Maintain control of airway in heavily sedated patients
Cardiovascular	Assess for dehydration (e.g., mucous membranes, skin turgor)	Assess intravascular volume status and cardiac function by bedside ultrasound
	Prevent or treat hypovolemic shock with oral or IV fluid administration	Administer IV fluids and vasopressors for refractory shock
	Limit medications that prolong QT interval (e.g., Haldol, quinolones)	Continuous monitoring with advanced life support for reversible conditions
Respiratory	Monitor for respiratory distress (e.g., rapid shallow breathing)	Monitor for respiratory complications by pulse oximetry and chest x-ray
	Avoid worsening pulmonary vascular leak with excessive fluid replacement	Administer oxygen and/or invasive ventilation for respiratory failure
Gastrointestinal	Administer antiemetic and antidiarrheal therapy	Treat vomiting and diarrhea and quantify gastrointestinal losses
	Monitor for ileus by exam and/or abdominal girth measurement	Monitor for ileus by measuring abdominal girth; ultrasound or x-ray evaluation
	Provide oral nutrition to avoid hypoglycemia	Provide enteral or parenteral nutrition with close laboratory monitoring
Genitourinary	Assess for renal insufficiency by urine frequency, volume, and color	Quantify total intake and urine output hourly
	Monitor and replace magnesium and potassium orally or intravenously	Monitor renal function and electrolytes frequently and replace as needed
	Avoid nephrotoxic agents	Avoid nephrotoxic agents; administer RRT for renal failure
Hematologic	Monitor for hematemesis or bloody stools as situation permits	Perform clinical and laboratory monitoring for bleeding and coagulopathy
	Administer phytonadione for bleeding, blood if available	Administer phytonadione and blood products (e.g., platelets, FFP) for bleeding
	Encourage ambulation to prevent deep venous thrombosis	Administer prophylactic anticoagulation with resolution of coagulopathy
Concurrent infections	Test for or empirically treat malaria	Test for and treat malaria if present
	Consider preemptive or therapeutic antibiotics	Integrate laboratory and radiologic data to assess potential bacterial infection

Abbreviations: PICC: peripherally inserted central catheter; CVP: central venous pressure; IV: intravenous; RRT: renal replacement therapy; FFP: fresh frozen plasma.

Loperamide has antisecretory and antimotility effects and has been effective in the treatment of diarrhea in Ebola patients (105, 106). However, antidiarrheal agents should not be used in the setting of ileus, obstruction, or inflammatory diarrhea, as manifested by blood or mucous in the stool. Serial measurement of abdominal girth may be performed in both low- and high-resource settings as a means of detecting intestinal obstruction or ileus. In high-resource settings, point-of-care abdominal assessment may also employ measurement of bladder pressure and the use of abdominal ultrasound and radiography. Oral, enteral, or parenteral nutrition may be required, depending on disease severity, associated complications (e.g., pancreatitis), and the care setting. Hypoglycemia must also be recognized and treated.

Intravascular volume depletion due to insensible losses from fever, limited oral intake, or vascular leak in the lungs or other organs may precede the onset of gastrointestinal symptoms, predisposing to renal insufficiency before the onset of shock. Dehydration should be recognized and managed early with oral or intravenous fluids. Oral rehydration solutions may be adequate in early or moderate

disease, assuming that vomiting can be controlled. Patients unable to drink fluids or those with evidence of shock require intravenous fluid replacement with isotonic solutions such as 0.9% normal saline. In high-resource settings, cardiac and intravascular volume status may be assessed by bedside ultrasound, and fluid-refractory shock can be managed with vasopressors. Close cardiac monitoring and periodic assessment of the QT interval is recommended, when possible. Advanced life support, including cardiopulmonary resuscitation for reversible conditions, should be considered in controlled settings with adequately trained staff.

An important aspect of fluid management is avoiding overly aggressive hydration, which may worsen pulmonary vascular leak and hypoxia. In clinical settings lacking access to oxygen or other types of supportive respiratory therapy, close monitoring for respiratory distress and careful fluid management are recommended. In high-resource settings, continuous monitoring of oxygen saturation and intermittent chest radiography facilitate the detection of pulmonary complications. Invasive mechanical ventilation may be required for multifactorial respiratory failure.

Renal function is also a critical aspect of Ebola virus disease, and careful monitoring of fluid intake and output are needed. Nephrotoxic agents, including nonsteroidal anti-inflammatory drugs and aminoglycoside antibiotics, should be avoided. In the early phase of illness or in moderately ill patients, electrolytes may be empirically replaced with oral solutions, but severe or ongoing loss requires frequent monitoring and replacement. Adequate magnesium replacement is required to achieve successful potassium supplementation. Renal-replacement therapy has been used in high-resource settings (158).

When conditions permit, clinically significant hemorrhage should be treated with phytonadione (vitamin K) and the administration of platelets, packed red cells, and fresh-frozen plasma, when available. Monitoring of coagulation parameters is recommended, and patients with severe disseminated intravascular coagulation with fibrinogen levels < 100 mg/dL should be given cryoprecipitate. Upon resolution of coagulopathy, pharmacologic prophylaxis of deep venous thrombosis with subcutaneous heparin or enoxaparin should be considered in nonambulatory patients.

Concurrent infections should be sought and treated. Given the high prevalence of malaria in settings where Ebola outbreaks occur, empiric treatment is recommended if malaria cannot reliably be ruled out. The choice of therapy should consider potential toxicities, including QT prolongation, the need for dosage adjustment with hepatic and renal dysfunction, and the ability to detect and treat toxicities in low-resource settings. Drugs used to treat chloroquine-resistant malaria that are associated with QT prolongation include quinine, quinidine, mefloquine, and artemether-lumefantrine. Secondary bacterial infections, including pneumonia and sepsis, often ascribed to gut translocation, are concerns in Ebola virus disease, but the optimal timing and choice of antibiotic therapy have not been established. In the absence of specific microbiologic testing capacity, preemptive antibiotic administration with coverage for enteric Gram-negative pathogens is reasonable.

Antiviral Therapy

The West African epidemic has markedly accelerated the development of antiviral therapy for Ebola and Marburg virus disease. By early 2014, a number of different drugs had been shown to prevent illness and death of filovirus-infected mice, guinea pigs, and nonhuman primates, but none had been administered to humans (159). As the outbreak expanded, several products were moved forward to Phase I trials and were also administered to accidentally infected medical workers from the United States or Europe under compassionate-use protocols. There were five types of these drugs: polyvalent antiserum, combinations of monoclonal antibodies (mabs), nucleoside/nucleotide analogues, interferons, and antisense molecules. Summaries of the current status of research and development efforts for Ebola therapies, vaccines, and diagnostic kits are available at the World Health Organization (136, 160).

Initial testing of polyvalent anti-Ebola serum produced in horses failed to achieve protection in macaques, and no evidence of a therapeutic benefit was found in macaques with convalescent whole blood (161–163). In contrast, a concentrated polyclonal IgG prepared from the pooled serum of macaques that had undergone vaccination and challenge with Marburg or Ebola virus was highly protective when administered to naïve macaques up to 3 days postexposure (164). Convalescent plasma has been administered to some patients in West Africa with uncertain effects (113) and one open-label study in patients in Guinea found no significant mortality benefit, compared to historical controls (165).

Initial testing of mab therapy of Ebola virus infection in laboratory animals did not appear promising; a single antibody targeting an epitope on the viral GP was protective in guinea pigs but not in macaques (166), but more recent evaluation of combinations of mabs targeting different epitopes on the virion surface GP has been very successful (167). A product containing three mabs, known as ZMapp®, was the first therapy to prevent the death of macaques when initiated after the development of illness (168). During the West African epidemic, Zmapp has been given under compassionate use to approximately 15 to 20 patients, mostly American or European health care workers, most of whom survived their infection (169). Whether these favorable outcomes were the result of mab therapy or simply reflect good supportive care in modern hospitals cannot be determined, especially as most patients received more than one experimental treatment. An open-label, randomized controlled trial comparing Zmapp therapy to supportive care is in progress (170).

Three nucleoside/nucleotide analogue antiviral drugs have also progressed from the laboratory to human use during the course of the West African epidemic. Favipiravir, a nucleoside analogue licensed in Japan for the treatment of novel influenza strains, was highly protective in Ebola virus-infected mice but only at dosage levels much higher than required for influenza therapy (32). Favipiravir has been administered therapeutically to several health care workers evacuated to the United States or Europe and in an open-label Phase 2 trial to a large cohort of African patients in Médecins Sans Frontières treatment centers in Guinea (136). Although the trial only compared treatment outcomes to previous patients who received no antiviral therapy, the findings suggested that favipiravir is beneficial if begun early in the course of illness in those with low viral loads (171). In contrast, no mortality benefit or antiviral effect was apparent in those with higher viral loads. A second nucleoside analog, BCX4430, which is highly potent in filovirus-infected macaques (35), has entered Phase I trials but has not yet been given to patients.

The third experimental product, GS-5734, a nucleotide analogue with a prodrug structure similar to that of the anti-hepatitis C compound sofosbuvir, is highly protective in

Ebola virus-infected macaques that had already developed signs of illness (172). It was used to treat a nurse who had recovered from Ebola virus disease but suffered a delayed recrudescence of infection in the central nervous system and also an infant in West Africa; both survived (125).

The broad antiviral activity of type I IFN suggests that it should inhibit filovirus infections, and limited therapeutic benefit has been observed in animal studies. Nonhuman primates treated daily with IFN-α2b, beginning on the day of virus challenge, showed a prolongation in mean time to death, and a regimen employing IFN-β had a similar outcome (162, 173). A clinical trial of IFN-β was initiated in Guinea; outcomes have not been reported (160).

Antisense oligonucleotides that bind to specific sequences in Marburg or Ebola virus messenger RNA have proven beneficial in laboratory animals but to date have only been given to a few Ebola patients. The small interfering RNA molecule TKM-Ebola, which targets mRNA encoding the viral polymerase and VP35, was reengineered from its original *Zaire ebolavirus* sequence to match the West African Makona strain and, when delivered in a lipid nanoparticle formulation, proved protective in rhesus macaques when treatment was begun on day 3 postinfection (34). TKM-Ebola was administered together with convalescent plasma to two accidentally infected American physicians (113), but a trial in Sierra Leone was terminated by its Data Safety and Monitoring Board because of lack of efficacy. Phosphorodiamidate oligonucleotides targeting specific filovirus mRNA sequences are also highly active in infected laboratory animals (174) and have undergone dose-escalating safety and pharmacology studies in humans (175), but their use for PEP or therapy in Ebola patients has not been reported.

Host-Directed Therapies

Efforts to develop effective treatments for filovirus disease have also focused on modification of damaging host responses to infection (160). One approach has been to prevent the initiation of DIC by blocking the binding of tissue factor to factor VIIa, using recombinant nematode anticoagulant protein C2 (rNAPC2) (176). When nine macaques were given daily injections of rNAPC2, beginning on the day of or the day after *Zaire ebolavirus* challenge, most showed a marked reduction in coagulopathy. Treatment also resulted in a striking decrease in peak serum levels of IL-6 and MCP-1 and a 100-fold drop in peak viremia in the three animals that survived infection. Treatment of Ebola virus-infected macaques with recombinant activated protein C also resulted in a survival benefit (177). Neither approach has been advanced to human trials. An attempt to treat Ebola patients in Sierra Leone with the host response modifier atorvastatin produced claims of therapeutic benefit that have not been documented (178).

CONCLUSION

The filoviruses were long considered a local problem in Central Africa, with little global significance (179). The massive Ebola epidemic in West Africa has broken that pattern, but although its scale is much larger than any previous outbreak, the disease itself and its mode of transmission appear to be unchanged from earlier outbreaks. Extensive spread was made possible by difficulties in recognizing the disease against a background of malaria and other febrile illnesses, and the severely limited medical, logistic and communications resources in one of the world's poorest regions. Importantly, when the virus spread to countries with more effective public health systems, such as Nigeria, chains of transmission were quickly detected and halted. Given the vastly increased awareness of Ebola virus disease, it is likely that future outbreaks will be recognized and contained quickly.

An important lesson from the current epidemic is that even in resource-limited settings, Ebola patients can receive meaningful treatment that reduces case fatalities. We have also learned that people infected with a deadly transmissible virus can be treated safely in modern hospitals in the United States and Europe, with a high likelihood of survival. The experience of managing large numbers of cases has reinforced the importance of early diagnosis so that fluid replacement can begin before the onset of shock. In severe cases, however, prevention of shock alone without adequate resources to diagnose and manage multiorgan failure may still result in death. Although a number of specific antiviral medications have been given to patients during the West Africa outbreak, their efficacy has not been established, and there is still a critical need for proven effective targeted therapies. Extensive experience has also been obtained with the recombinant adenovirus and VSV Ebola vaccines. The latter was shown effective in a ring vaccination trial, providing great promise as an emerging tool to protect medical personnel and control the spread of disease in future outbreaks.

Perhaps the most important lesson from the West African epidemic is that doctors and nurses can be counted on to stay at their posts, caring for Ebola patients at the risk of their own lives. In Guinea, Liberia, and Sierra Leone more than 800 health care workers became infected, and at least 500 died. The support of the industrialized countries to create and maintain an effective public health system in West Africa, capable of responding both to a filovirus outbreak and to the range of more common endemic diseases, will be their best memorial.

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Influenza Virus

FREDERICK G. HAYDEN AND PETER PALESE

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"If influenza is a riddle wrapped in mystery inside an enigma, then the viral genes are the riddle, the variable surface antigens for which they code are the mystery, and the course and cause of epidemics the ultimate enigma."

E. Kilbourne, 1980.

Influenza viruses are unique among the respiratory viruses with regard to their frequent antigenic changes, seasonality, and impact on the general population. They can cause explosive outbreaks of febrile respiratory illness across all age groups and often substantial mortality, particularly in aged and chronically ill persons. Epidemics resembling influenza have been recorded since antiquity. The plague of Athens in 430 to 427 BC, described by Thucydides, has been postulated to have been due to epidemic influenza complicated by toxigenic staphylococcal disease (1). The greatest effects of influenza are seen when novel strains, to which most persons are susceptible, cause worldwide outbreaks, or pandemics. The most profound of these in modern times was the 1918 pandemic that may have claimed as many as 100 million lives worldwide (2). Sequencing of RNA fragments from tissue samples taken from 1918 pandemic victims enabled reconstruction of the extinct 1918 virus and study of its virulence in animal models (3, 4).

Influenza virus was first isolated from chickens with fowl plague in 1901. Swine influenza virus, possibly transmitted to swine from humans during the 1918 pandemic, was isolated initially in 1931, and the first human influenza A virus was recovered in 1933 (Fig. 1, Fig. 2). Recognition of antigenic variation, growth in embryonated eggs, and the ability of virus to agglutinate erythrocytes followed shortly. These discoveries provided reliable means of virus isolation and serologic testing. Influenza B and C viruses were first isolated in 1940 and 1947, respectively. Recent studies identified a new genus, provisionally designated influenza D, with reservoirs in pigs and cattle (5, 6).

During the 1940s, crude inactivated vaccines were introduced, and these were followed by more purified, less reactogenic ones. In 1948, WHO established an influenza surveillance program that continues to monitor global activity and to facilitate strain selection for vaccines. Among the first antiviral agents, amantadine and rimantadine were shown to have prophylactic and therapeutic activity for influenza A in the mid-1960s; the neuraminidase (NA) inhibitors followed in the late 1990s. Despite the availability of improved vaccines and antivirals, seasonal influenza remains an important public health problem. The annual burden of interpandemic influenza in the United States has averaged approximately 20 million respiratory illnesses, over 200,000 hospitalizations, and between about 3,000 to 49,000 deaths in recent years (7). The first pandemic of the 21st century, due to a swine-origin virus emerging initially in North America, rapidly spread globally but was fortunately less severe than predicted. However, emerging zoonotic influenza viruses, like avian A(H5N1) in 1997 and again 2003, swine variant A(H3N2) in 2012, and avian A(H7N9) in 2013, continue to cause outbreaks and pose potential pandemic threats (Table 1).

VIROLOGY

Classification

Influenza A, B, and C viruses belong to the family *Orthomyxoviridae*, which is characterized by a segmented, negative-strand RNA genome (8). The influenza A and B virus types each carry eight different RNA segments, whereas the influenza C virus genome has seven. Infections by strains of all three influenza types (genera) can be associated with classical influenza symptoms in humans. The fourth genus of the *Orthomyxoviridae* consists of the Thogoto viruses. Two of its six members, Dhori and Thogoto viruses, are tick-borne agents that have caused human infections, including one in the United States by a novel Thogoto virus tentatively called Bourbon virus (9). The fifth orthomyxovirus genus is made up of the isaviruses (infectious salmon anemia virus), and recently a sixth genus was added, Quaranfil virus. A novel influenza C-like virus has been proposed as a new D genus because it shares approximately 50% identity with influenza C, and, unlike members of the same genus, it fails to yield infectious progeny during reassortment studies with influenza C (5).

The influenza A, B, and C virus types were defined originally by the observation that antisera made against the core proteins of a specific strain cross-reacted only with those

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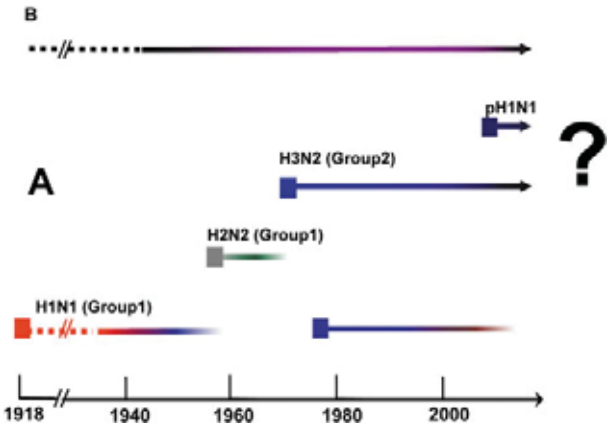

FIGURE 1 Circulation of human influenza A and B viruses. Solid lines indicate that strains have been isolated and characterized. Three different influenza A virus HA subtypes (H1, H2, and H3) and two NA subtypes (N1 and N2) were identified in humans over the last 100 years. The RNA genome of the extinct pandemic 1918 virus has been sequenced from RNA fragments in formalin-fixed and frozen tissue samples from 1918 victims following RT-PCR (3). The virus was then reconstructed in the laboratory by reverse genetics (solid square) (4).

belonging to the same type and not with those of another type. Sequencing studies have confirmed this classification scheme, showing that genes coding for the matrix protein (M1) or the nucleoprotein (NP) of strains belonging to one type are more closely related to each other than to the corresponding genes of strains from different influenza virus types; however, nucleotide-sequence comparisons indicate that all influenza virus types share a common ancestor (8). Low intertypic (20 to 30%) and high intratypic (>85%) identity exists for the M1 and NP proteins.

Subtypes occur only among the influenza A viruses. The extent of serologic cross-reactivity for the surface glycoproteins, the hemagglutinin (HA) and the NA, was previously used to differentiate them; more recently, sequencing of the corresponding genes has been used to differentiate them. Based on sequence analysis, 18 distinct HA and 11 distinct NA subtypes are now recognized, but only 3 HA subtypes (H1, H2, and H3) and 2 NA subtypes (N1 and N2) are known to have caused extensive outbreaks in humans (Fig. 1, Fig. 2). Virus strains recovered from humans are named on the basis of type, location of isolation, serial number from that location, year of isolation, and, in the case of influenza A viruses, the subtypes of the HA and NA antigens (e.g., A/Switzerland/9715293/2013 [H3N2]).

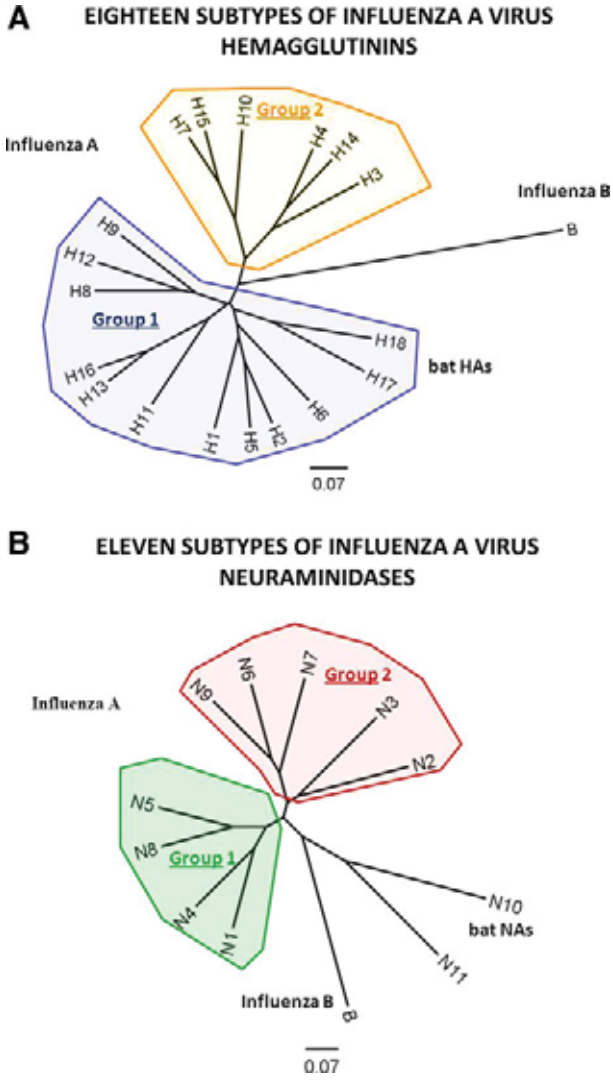

FIGURE 2 Phylogenetic tree of influenza virus hemagglutinins (HA) (**A**) and influenza neuraminidase (NA) (**B**). Twelve HA subtypes, including two bat HAs, make up group 1 (blue) and 6 HA subtypes make up group 2 HAs (orange). There is only one influenza B virus HA type (no subtypes). Four NA subtypes make up group 1 (green) Influenza A virus NAs, and 5 are in group 2 (red). Influenza A virus NA. The bat NAs (N10 and N11) and the influenza B virus NA (no subtypes) are evolutionarily divergent. Scale represents a 7% change in amino acid differences. Courtesy of Krammer (486), reprinted with permission.

Composition

Influenza viruses are spherical lipid-containing viruses with a diameter of approximately 100 nm (Fig. 3A). Filamentous forms of the virus can also be observed with electron microscopy, appear to be infectious, and are postulated to be predominant particles during productive infection in the lungs. The activities and functions of the viral structural and nonstructural (NS) proteins are summarized in Table 2. The virus surface is covered by HA and NA glycoprotein spikes, the structures of which have been resolved by X-ray diffraction (10, 11). A small number of molecules of the M2 protein are also found in the membrane of the virus particle. Below the lipid membrane is a layer of the M1 protein surrounding the ribonucleoprotein (RNP) core. This core consists of the eight RNA segments, which are associated with one to several copies of the viral polymerase complex (PB1, PB2, and PA) proteins and are covered by viral NP molecules.

Surface Proteins

The HA is a trimeric rod-shaped spike with a hydrophobic carboxy terminus anchored in the viral envelope and the hydrophilic end projecting outward from the virus. The exposed portion contains the antigenic domains and binding sites for sialic acid (N-acetylneuraminic acid) residues in receptors of host cells or erythrocytes (Fig. 3B). The HA facilitates both attachment of the virus to host cell receptors and penetration of the virus. Posttranslational proteolytic cleavage into HA1 and HA2 by a serine protease is essential

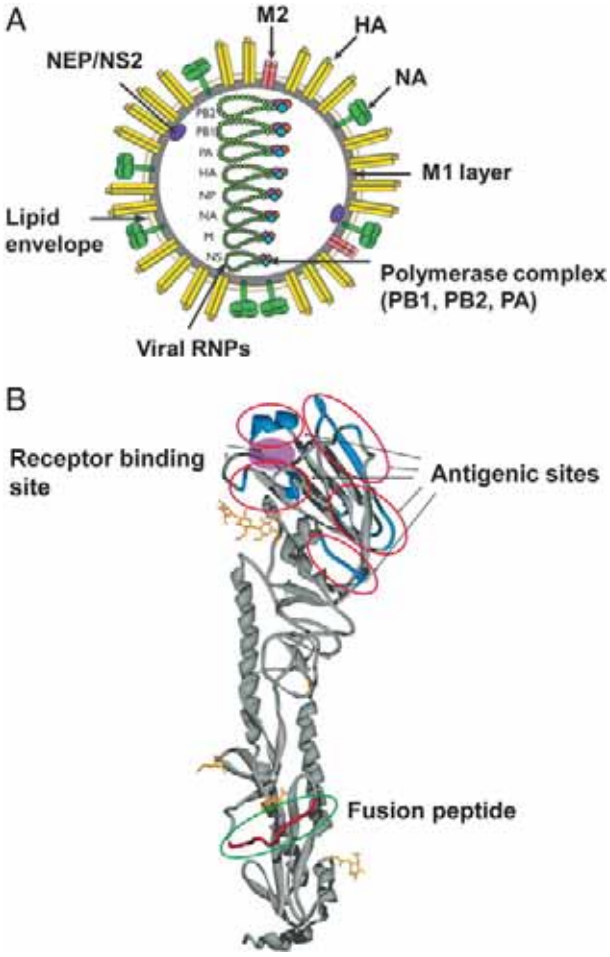

FIGURE 3 (A) Schematic diagram of an influenza A virus. HA, NA, and M2 are transmembrane proteins anchored in the lipid membrane of the virus. Inside the lipid membrane is a layer of the M1 protein. The RNP core consists of the different RNA segments, which are covered by NP molecules. In addition, they carry an RNA-dependent RNA polymerase complex (P proteins). The 3′ and 5′ ends of the RNA segments are stabilized by the polymerase complex into panhandle structures. NEP is also part of the viral core structure. (B) Schematic depiction of influenza virus HA. Antigenic changes cluster into five highly variable regions surrounding the receptor-binding pocket (purple oval). Courtesy of James Stevens and Ian Wilson (487), reprinted with permission.

for infectivity. Certain avian HAs (H5 and H7) contain extra basic amino acids in the HA cleavage site, which allow for activation by cellular proteases widely distributed in tissues and result in systemic replication in birds and sometimes mammalian hosts (12). These so-called highly pathogenic avian influenza (HPAI) viruses have caused multiple outbreaks in poultry, and, recently, sporadic disease in humans (Table 3). The HA2 portion is principally responsible for fusion of the virus envelope and cell membrane, whereas the HA1 portion contains the binding sites for host-cell receptors, as well as at least four major antigenic domains. The overall configuration of the HA and its functions are conserved during virus evolution, but frequent amino acid substitutions occur in the antigenic sites. Antibodies to the head domain of HA can prevent attachment of the virus to cells, and antibodies to the stalk domain of HA can also inhibit virus replication by blocking fusion.

TABLE 1 Influenza-related websites of interest

United States Centers for Disease Control and Prevention (USCDC): Influenza
http://www.cdc.gov/flu/
USCDC Weekly Influenza Activity
http://www.cdc.gov/ncidod/diseases/flu/weekly/htm
World Health Organization (WHO): Influenza
http://www.who.int/topics/influenza/en/
WHO human-animal interface web page
http://www.who.int/influenza/human_animal_interface/en/
European Centers for Disease Control: Influenza
http://ecdc.europa.eu/en/healthtopics/influenza/Pages/home.aspx
World Organisation of Animal Health (OIE) web page: Web portal on Avian Influenza
http://www.oie.int/animal-health-in-the-world/web-portal-on-avian-influenza/
Food and Agriculture Organization of the UN (FAO) webpage: Avian Influenza
http://www.fao.org/avianflu/en/index.html
United States Department of Agriculture Animal and Plant Health Inspection Service
https://www.aphis.usda.gov/wps/portal/aphis/home

NA is a mushroom-shaped tetramer that is anchored in the lipid envelope at its amino terminus (13). It cleaves terminal sialic acid residues from various glycoconjugates and plays an essential role in release of virus from infected cells and the spread within the respiratory tract. By removing sialic acid residues from the virion envelope and cell surface and from mucins present in respiratory tract secretions, the NA activity (receptor-destroying enzyme [RDE]) prevents aggregation of viral particles and may enable penetration of virus through respiratory secretions (8). Anti-NA antibodies and NA enzyme inhibitors prevent release of virus from infected cells. A balance of HA and NA activities is essential for efficient virus replication.

The M2 protein, a homotetramer present only in influenza A viruses, appears on the surface of infected cells and is also incorporated into virions as a third integral membrane protein. The ion channel activity of M2 plays a role in uncoating of virus in endosomes and possibly in regulation of virus assembly. Amantadine and rimantadine inhibit the ion channel activity of M2. Antibody to M2 is associated with reduced viral replication and heterosubtypic protection in animals. The ectodomain of M2 (M2e) is highly conserved and has been targeted by both investigational monoclonal antibodies (14) and vaccines.

The single glycoprotein of influenza C virus encompasses receptor-binding, membrane-fusing, and receptor-destroying activities. It is directed against 9-O-acetyl-N-acetylneuraminic acid, a receptor different from N-acetylneuraminic acid, which is recognized by influenza A and B viruses (8).

Genome

Influenza A and B viruses each possess eight different RNA segments that can code for at least 11 different proteins (Fig. 4A). Influenza C and D (proposed) viruses lack an NA gene and thus have only seven RNA segments that code for at least nine different polypeptides. Dhori and Thogoto viruses possess six and seven RNA segments, respectively. In nature, a reassortment of gene segments after co-infection of

TABLE 2 Influenza A virus RNA segments and proteins

RNA segment	Protein	Protein size (amino acids)	Functional activity(ies)
1	PB2	759	Cap recognition
2	PB1	757	RNA polymerase, elongation
	PB1-F2	87	Proapoptotic activity, IFN antagonist
3	PA	716	RNA polymerase subunit,
	PA-X	∽260	proteolysis, endonuclease activity
			Modulation of host response
4	HA	∽560	Attachment to receptors, fusion of membranes
5	NP	498	Structural component of RNP, nuclear import of RNA
6	NA	∽450	NA/sialidase activity, release of virus
7	M1	252	Structural protein, nuclear export of RNA, viral budding
	M2	96	Ion channel
8	NS1	∽230	Interferon antagonist, multifunctional protein
	NEP (NS2)	121	RNP nuclear export, regulation of RNA synthesis

cells by human and animal influenza A viruses may be responsible for the emergence of new pandemic strains (see following paragraphs and Fig. 5). However, reassortment of genes among viruses belonging to different influenza virus types has not been observed. Apparently, the proteins of different virus types have evolved sufficiently to preclude replication of intertypic reassortants. Homologous recombination between corresponding RNA segments of different influenza viruses has not been observed, in contrast to the high frequency of recombination observed among the genomes of retroviruses or of positive-sense RNA viruses, like polioviruses. Influenza A virus isolates obtained during a single outbreak are variable in genetic sequence. This genetic heterogeneity provides a basis for evolutionary adaptation and the ability of the virus to cope with selective immunologic and drug pressures.

Influenza A viruses code for approximately 13,600 nucleotides (nt), influenza B viruses for 14,600 nt, and influenza C viruses for approximately 12,900 nt (15). A genomic map of an influenza A virus is shown in Fig. 4. Each RNA codes for a different protein, with the two smallest RNA segments each transcribing an additional spliced mRNA.

Biology

Replication Strategy

Influenza A and B viruses adsorb to receptors on the cell surface, which contain sialic acid (8) (Fig. 6). It is not known which specific carbohydrate-containing membrane proteins (or, less likely, glycolipids) are the major targets for the initial binding. Human influenza viruses preferentially attach to sialic acid with an α(2,6) linkage to galactose-containing oligosaccharides, whereas avian and equine viruses prefer α(2,3) linkages. Influenza C virus binds to 9-O-acetyl-N-acetylneuraminic acid-bearing receptors. After internalization into endosomes, the cleaved HA undergoes an acid pH-triggered conformational change into a fusogenic form. This event facilitates the fusion of the viral and endosomal membranes. The ion channel in the viral membrane, comprised of M2 polypeptides, is also activated by the acid pH in the endosomes. This process results in an influx of protons into the virion, which probably loosens the

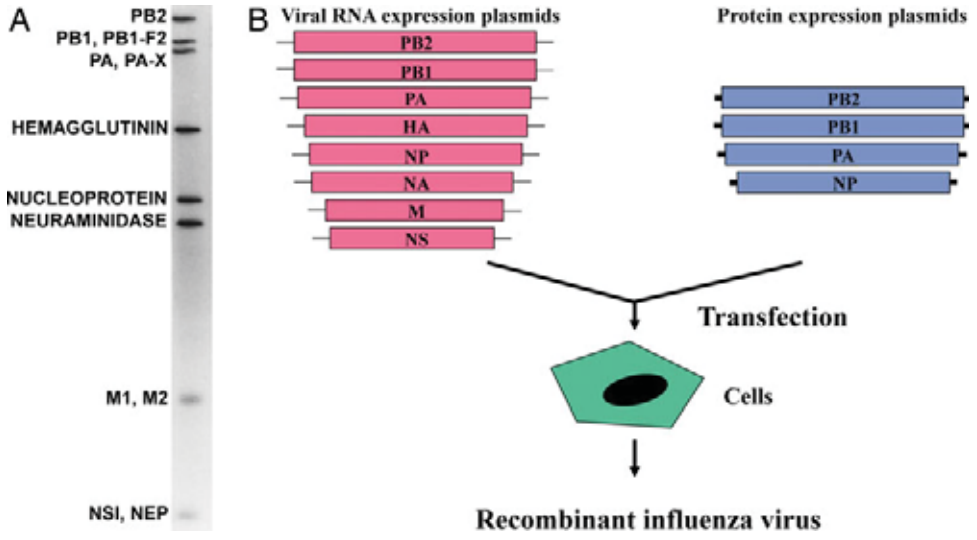

FIGURE 4 Reverse genetics of influenza viruses. (A) Genetic map of influenza A/PR/8/34 (H1N1) virus. Purified RNAs were separated on a polyacrylamide gel; assignment of genes coding for one or two viral proteins is indicated. (B) Plasmid-only rescue of infectious influenza virus. Twelve plasmids are introduced into mammalian cells: four plasmids lead to expression of the viral proteins required for viral RNA replication (PA, PB1, PB2, and NP), and eight plasmids express precise copies of the eight viral RNA segments (PA, PB1, PB2, HA, NP, NA, M, and NS). The resulting viral RNAs are replicated and transcribed by the reconstituted influenza virus RNA-dependent RNA polymerase. Recombinant infectious influenza virus is generated 48 to 72 hours after transfection of cells (26, 27). Recently, several improvements of the plasmid-only rescue for influenza A viruses have been introduced (28, 29). Also, reverse-genetics systems for influenza B and C viruses have been successfully developed (28, 30–32, 488).

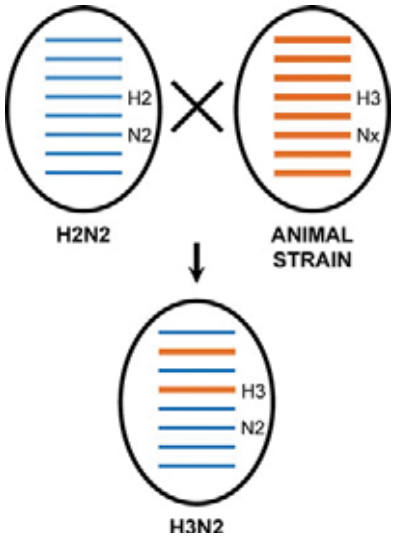

FIGURE 5 Reassortment of influenza A viruses leading to a new pandemic strain. Co-infection of the same cell with a human A(H2N2) (thin blue lines) virus and an animal (avian) strain with an H3 HA (thick orange lines) results in reassortment. The A(H3N2) virus responsible for the pandemic in 1968 is postulated to derive its PB1 and H3 genes (thick lines) from the animal strain and its remaining six RNAs, including the N2 gene, from the A(H2N2) parent (thin lines) (12, 489).

M1 protein from the RNP core and ultimately facilitates RNP release into the cytoplasm (uncoating). The RNA segments of the incoming virus particle (vRNA) remains associated with viral protein throughout the uncoating process and enters the nucleus as RNP through the nuclear pore complex (16).

Following uncoating of the virus and transportation of the RNP into the nucleus, transcription and replication of the viral genome take place in the nucleus. The recently reported X-ray diffraction analysis of the full polymerase complex, consisting of PB1, PB2, and PA proteins, shows the precise location of the cap-binding and nuclease activity in the polymerase complex, as well as those of the domains allowing for RNA elongation and poly(A) addition (17, 18). The incoming viral RNP is the template for the viral RNA-dependent RNA polymerase, which produces two different species of viral RNA: (i) an mRNA that derives its first 9 to 15 nt from host mRNA, is capped at the 5′ end, and carries a poly(A) tail lacking the 3′ 15 to 16 nt of the template RNA, and (ii) a full-length complementary copy (cRNA) of the template RNA. This cRNA itself becomes a template for the amplification of viral RNAs, leading to additional copies of vRNA (Fig. 7). The minimal promoter sequences of the vRNA and the cRNA molecules appear to be the highly conserved 3′ sequences. In addition, the 5′ sequences of the templates are part of the transcription-replication complex.

mRNA synthesis is regulated, but control of the switch from transcription (mRNA synthesis) to replication (amplification of vRNA) is still not well understood. Although the different vRNA molecules appear to be equimolar in the virus and in infected cells, the mRNAs of the HA, M, NP, and NS genes are much more abundant than those of the three polymerase (P) genes or of the NA gene. Also, mRNA synthesis is down-regulated late in the replication cycle, while vRNA synthesis continues. Finally, protein expression is affected by several additional mechanisms: (i) for the M and NS genes, partial splicing of their mRNAs leads to the expression of two viral proteins from each of these RNA segments; (ii) coupled stop-start translation of tandem cistrons gives rise to two proteins, the M and the BM2 proteins, from the M segment of influenza B viruses; (iii) ribosomal initiation from AUG codons located in different reading

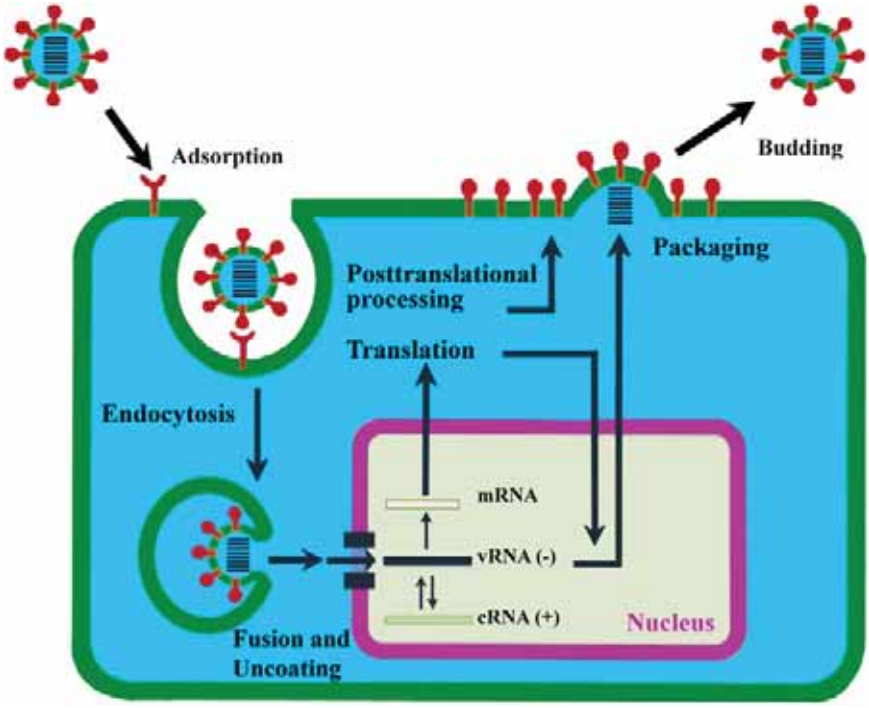

FIGURE 6 General replication scheme of influenza virus. For a description of different replication steps, see the text.

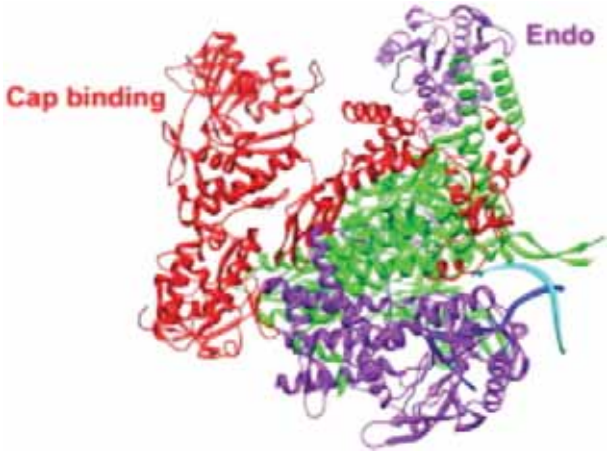

FIGURE 7 RNA transcription/replication by influenza virus polymerase. Structure of the influenza A virus (bat) polymerase from Ortin and Martin-Benito (490) according to Pflug et al. (17). The PB1 (green), PB2 (red), and PA (magenta), and the cap binding (part of PB2) and endonuclease (part of PA) domains are indicated. The synthesis of viral mRNA is initiated by cap-snatching, which involves the binding of the cap of a host RNA followed by an endonuclease (PA) cleavage. The host mRNA-derived capped RNA primer is then used to allow the RNA synthesis by the PB1 domain of the viral polymerase complex (491).

frames on the PB1 gene of influenza A viruses and on the NA gene of influenza B viruses leads to the expression of two additional proteins, PB1-F2 and NB, respectively (in addition to the PB1 and NA proteins of the corresponding RNAs) (8, 19); and (iv) ribosomal frame shifting in the case of PA-X. The latter is a fusion protein with the C-terminal portion derived from a second open reading frame of the PA segment (20).

Assembly and packaging of RNAs into infectious viruses involve several cellular compartments. The viral P proteins and the NP have specific nuclear localization signals, so they can travel into the nucleus, where they associate with viral RNAs to form RNPs. The nuclear export of these RNPs likewise requires a unique mechanism and depends critically on the presence of the M1 protein and nuclear export protein (NEP) (21, 22). M1 binds to viral RNPs in the nucleus and promotes their export by interacting with NEP, which possesses a nuclear export signal and the ability to interact with M1 (22, 23). The virus-induced Raf/MEK/ERK signal cascade is essential for efficient replication, and blockade of this pathway slows viral RNP export and reduces viral titers (24).

Following the export of the RNPs into the cytoplasm, they assemble at the cytoplasmic membrane under patches of the viral glycoproteins HA and NA. M1 plays a critical role in the assembly and budding of infectious virus. Virus particles bud from the cytoplasmic membrane, while the NA clears the virus and the cell membrane of sialic acids to prevent virus-virus aggregation and virus-cell surface retention, respectively. Once the virus particle is outside the cell, the NA may further help to remove sialic acids from mucous substances in the respiratory tract, thus allowing the virus to reach other epithelial cells (8).

The packaging of vRNAs into influenza virus particles most likely involves a specific packaging mechanism: the 3′ and 5′ noncoding regions of the vRNA segments contain cis-acting signals, which are required for packaging the vRNAs into virus particles. In addition, sequences in the coding regions of the influenza A virus RNAs contribute to the efficient packaging of just the eight different segments in virus particles (8). Thus, packaging is not random, although the precise mechanism by which the packaging of a full complement of just eight RNAs occurs remains to be fully elucidated.

Genetic Engineering of Influenza Viruses

The genome of negative-sense RNA viruses is not infectious, and thus direct transfection of cDNA-derived influenza virus RNAs does not result in the formation of infectious virus particles. However, systems have been developed which allow the *in vitro* reconstitution of biologically active RNP from synthetic RNAs and purified viral proteins. Transfection of synthetic RNPs into helper virus-infected cells allowed the rescue of transfectant viruses which contain one or more genetically engineered segments (25). Helper virus-free systems for producing recombinant influenza A viruses have been developed (26, 27) (Fig. 4B). Plasmids are transfected into cells, and infectious virus can be generated within 48 to 72 hours. In these plasmid-only systems, the vRNAs, as well as the viral RNA polymerase, are generated from the transfected plasmids. Improvements in this technology now make it easy to do structure-function analysis of individual viral proteins and have opened the way for the construction of improved influenza virus vaccines and influenza virus vectors (28, 29). Reverse-genetics systems have also been successfully developed for influenza B and C viruses (30–32).

Host Range

Type A influenza viruses have a broad host range (33, 34). Wild avian species, particularly ducks and other aquatic waterfowl, are the primary reservoir and harbor viruses expressing the 16 (non-bat) HA and 9 (non-bat) NA subtypes. In avian species, influenza viruses replicate in both the respiratory and gastrointestinal tracts, and there can be shedding of high quantities of virus in the feces. Infectious virus is readily recovered from cold lakes and other water sources frequented by birds. In contrast to the progressive changes of human viruses, avian viruses generally show little antigenic variation within the same subtype, although the rapid evolution and antigenic variation of HPAI A(H5N1) viruses is an exception (35). Thus, aquatic birds represent an enormous migratory reservoir of influenza A virus genes that can be potentially incorporated into influenza viruses transmitted to other species (12).

Recently, the RNAs of two new HA (H17 and H18) and of two new NA (N10 and N11) subtypes have been identified in bats, but no infectious viruses with these new subtypes have been isolated so far (36). Vesicular stomatitis virus, pseudotyped with the surface glycoproteins from bat influenza viruses, efficiently infect bat cell lines but not those derived from primates or swine and do not attach to sialic acid receptors, findings that suggest low potential for zoonotic infection (37).

The principal natural hosts of mammalian influenza A viruses are humans, swine, and horses (H7N7 and H3N8 subtypes). Outbreaks have also been documented in other species, including marine mammals (whales and seals), mink, dogs, and ruminants (e.g., reindeer). The HPAI A (H5N1) virus has caused severe disease in felids although not swine. Influenza A(H3N8) subtype virus infections in dogs represents a species jump from horses (38), and receptors recognized by equine influenza viruses are present on

canine respiratory epithelial cells (39). Both equine and dog influenza viruses are endemic in the United States, but, to date, in contrast to swine influenza viruses, they have not been recognized to infect humans.

The fact that influenza A viruses populate nonhuman species has important epidemiologic consequences. First, human and nonhuman influenza A reassortant viruses may lead to new pandemic viruses (see Fig. 5 and below). Second, the presence of an animal reservoir enables the virus to continue to circulate outside the human population, so eradication of human influenza A by immunization of humans is unlikely. Influenza B and C viruses are primarily pathogens of humans, but influenza B virus may infect seals, dogs, cats, and possibly swine. However, widespread animal-to-animal or human-to-animal transmission of influenza B viruses has not been identified to date. Influenza C viruses have been isolated from swine. Recently identified influenza D (proposed) viruses can cause respiratory illness in pigs and cattle (5, 6, 40). Full explanations do not exist currently for the host-range characteristics of the different influenza viruses. The virulence and host range of influenza viruses relate to the surface glycoproteins, as well as to other viral proteins (8).

Experimental infection has been accomplished in a variety of species, including hamsters, cotton rats, horses, and nonhuman primates, but mice, ferrets, guinea pigs, chickens, and swine are most commonly used. Pigs can be experimentally infected with avian influenza viruses representing nearly all HA subtypes. Ferrets can be infected with non-adapted human influenza viruses and serve as a useful model for studying viral virulence and transmission (4). A useful transmission model has also been developed using guinea pigs (41).

Growth in Cell Culture

Traditionally, influenza viruses have been grown in embryonated chicken eggs, both for laboratory purposes and for vaccine production. Primary cell cultures, including African green and rhesus monkey, hamster, bovine, or chicken kidney cells, and established cell lines like Madin-Darby canine kidney (MDCK) and mink lung epithelial cells, can be used to replicate influenza viruses (42). Propagation in non-primary cell lines lacking the protease needed for HA cleavage requires the addition of trypsin in serum-free medium. Hemagglutination by chicken, turkey, horse, or human erythrocytes remains a frequently used tool to identify and measure the replication of influenza viruses in eggs and cell culture. Future influenza vaccine manufacturing will rely increasingly on the growth of virus in cell culture to avoid the cumbersome use of embryonated eggs, and various cell systems are being developed or already used for vaccine virus production (e.g., MDCK, Per.C6, EB66, and Vero).

Inactivation by Physical and Chemical Agents

Influenza virus proteins and RNAs can be readily inactivated by ionizing radiation, high (>9) or low (<5) pH, and temperatures above 50°C. The stability of the virus depends on the surrounding medium, its protein concentration, and its ionic strength. Influenza viruses are enveloped viruses and thus are susceptible to agents that which affect membranes, including ionic and nonionic detergents, chlorination, and organic solvents (43). At 4 °C in phosphate-buffered saline solution containing physiological protein (albumin) concentrations, influenza viruses can be stable for months. On the other hand, drying of viral suspensions inactivates the virus in less than 12 hours on porous environmental surfaces and in 24 to 48 hours on nonporous environmental surfaces (44, 45). The virus can remain infectious for 24 hours or more after aerosolization under conditions of low (25%) or high (80%) relative humidity but is less stable under conditions of intermediate (50%) relative humidity (46). Environmental persistence studies indicate that influenza virus retains infectiousness longer at low temperatures, in water compared to in air, and in fresh, compared to salt, water (47).

EPIDEMIOLOGY

Influenza viruses have a worldwide distribution and cause outbreaks with variable intensities annually. Rapid onset and dissemination of infection are characteristic features of epidemic influenza. These relate to a short incubation period, which averages 2 days and ranges from 1 to 5 days, and to high concentrations of virus in respiratory secretions during the initial phase of illness (48). Influenza surveillance information is available through the CDC and WHO websites (Table 1).

Transmission

Influenza viruses spread from person to person by airborne droplets expelled during coughing, sneezing, or speaking. The relative efficiencies of different exposure routes (droplet, short-distance small-particle aerosols, hand contamination with self-inoculation following direct or fomite contact) are uncertain and likely vary with epidemiologic and environmental conditions (49, 50). Zoonotic influenza virus infections (discussed below; Table 3) are also spread by these routes through direct and indirect exposures and perhaps rarely by oral ingestion.

In volunteers experimentally infected with a human virus, approximately 10- to 100-fold-smaller viral inocula are needed to initiate infection after small-particle aerosol exposure of the lower respiratory tract than after intranasal inoculation of the upper respiratory tract. The human infectious dose is estimated to be 1 to 5 50% tissue culture infectious doses following experimental small-particle (1 to 5 μm) aerosol exposure (49). Intervention studies with topically delivered antivirals, like zanamivir and interferons (51), indicate that natural infection by human influenza viruses is initiated most commonly in the pharynx or tracheobronchial tree. Aerosol transmission has been implicated in both household (52) and closed outbreaks, where clinical attack rates may exceed 70% after common-source exposure in confined spaces (53). Influenza virus RNA is readily detected on fomites (44), and virus retains infectiousness longer on hard, nonporous surfaces, in low humidity, and at cooler temperatures (41, 45, 54), but the importance of transmission via fomites is uncertain. In acute care settings, small-particle aerosols containing influenza virus RNA are detected at decreasing concentrations within 6 feet from the patient's head during routine patient care (55), and aerosol-generating procedures have been implicated in nosocomial transmission (56). In households, upper respiratory tract viral loads and symptom severity in the index case, contact susceptibility, and social behaviors affect the risk of transmission (57). Deep sequencing studies indicate that most infections in contacts are acquired within the household, many index cases harbor mixed lineage infections that are transmissible, and an estimated 90 to 250 virus particles appear to be required for productive multilineage infection in contacts (58).

TABLE 3 Examples of human illness due to infection by avian influenza viruses[a]

Years of reported detection	Locations	Influenza A subtype	Cumulative no. of laboratory-confirmed human illnesses (to April, 2016)	Primary syndromes/comment
1959, 1977, 1980, 1996	USA, Australia, UK	H7N7 (LP)	∽5	Conjunctivitis
1997	Hong Kong SAR	H5N1 (HP)	18	Pneumonia; six deaths
1999-ongoing[b]	Hong Kong SAR, China, Egypt, Bangladesh	H9N2 (LP)	28	ARI, ILI; circulating in poultry in parts of Africa, Asia and Middle East
2002, 2007	USA, UK	H7N2 (LP)	2	ILI
2003-ongoing[b]	Multiple countries in Asia, Middle East, eastern Europe, and Africa (exported case to Canada)	H5N1 (HP)	850	Pneumonia, ARDS, multiorgan failure; mortality >50%. Limited, nonsustained human-human transmission to date[b]
2003	The Netherlands	H7N7 (HP)	89	Conjunctivitis, ILI; 1 fatal pneumonia. Limited human-human transmission likely.
2003	USA, UK	H7N2 (LP)	5	ARI in immunocompromised host; ILI, conjunctivitis
2004, 2010	Egypt, Australia	H10N7 (LP)	2	ILI
2003, 2004, 2006, 2012	Canada, Mexico, UK, Italy	H7N3 (LP)	3	Conjunctivitis; seropositivity
		H7N3 (HP)	4	
2013-ongoing[b]	China (exported cases to Malaysia, Canada)	H7N9 (HP)	770	Pneumonia, ARDS; mortality >35% in hospitalized; ARI, ILI; Limited, nonsustained human-human transmission to date[b]
2013	China	H10N8 (LP)	3	Pneumonia/ARDS, 2 fatal cases
2013	Taiwan	H6N1 (LP)	1	ILI
2014- ongoing[b]	China	H5N6 (HP)	14	Pneumonia/ARDS, mortality >35%. Circulating in poultry in China, Laos, Vietnam

[a]Abbreviations: LP, low chicken pathogenicity; HP, high chicken pathogenicity; ILI, influenza-like illness; ARI, acute respiratory illness (not specified).
[b]Consult the WHO and USCDC websites (Table 1) for updated information.

Airline travel plays a role in long-range dissemination of influenza (59), and high rates of virus movement occur internationally between urban centers (60). One hypothesis, that epidemic spread involves long-range atmospheric transmission of aerosolized influenza virus (61), remains unproven, although viral RNA detection has been reported in airborne dust samples (62), and wind dispersal may contribute to spread of avian influenza viruses between farms (63).

Antigenic Change

The changing antigenicity of influenza viruses enables continued circulation in human populations and makes their behavior unpredictable (64). Relatively minor changes, called antigenic drift, result from stepwise point mutations in the gene segment coding for the HA or NA. Antibody-driven sequential amino acid changes occur in antigenically important regions over time; this process sometimes requires concurrent changes in both glycoproteins for maintenance of viral fitness (65). In the case of influenza A virus, these result from the selective pressure of increasing levels of population immunity and lead to the emergence of epidemiologically important drift variants every 2 to 3 years. Amino acid substitutions in HA and NA have been observed at rates of approximately 0.5 to 1% per year over a two-decade period. Changes occur predominantly in the HA1 peptide and are distributed over the surface of the molecule, as well as clustering into five hypervariable regions (Fig. 3B). Viral lineages undergoing the greatest number of mutations in a restricted number of rapidly evolving HA1 codons appear to be the progenitors of future epidemic strains (66). Drift strains of epidemiological importance usually have substitutions in two or more of the antigenic sites of the HA and arise when such mutations lead to substantial antigenic change (67). Such variants are able to spread because of larger numbers of susceptible individuals and have a higher likelihood of causing symptomatic infections. Antigenic variation has occurred more rapidly in H3 than H1 subtype influenza A viruses and is less pronounced in influenza B and C viruses. Multiple evolutionary lines of influenza B or C viruses may co-circulate. Viruses representing two influenza B virus lineages, designated B/Victoria/2/87 and B/Yamagata/16/88, have circulated in various proportions in recent years. Seasonal influenza viruses undergo frequent gene reassortments that contribute to their evolution and genetic diversity (68). New antigenic variants of public health concern are usually identified by monospecific antisera raised in animals, typically ferrets, and by convalescent-phase sera from persons immunized with vaccines containing previous strains.

For influenza A viruses, marked changes in HA, with or without change in NA, called antigenic shift, are due to the acquisition of new gene segments. This may occur during

TABLE 4 Influenza A virus pandemics and related influenza events since late 19th century

Year of appearance	Duration of circulation (yr)	Virus subtype	Common designation	Estimated mortality in the United States	Comment
1889	?28	H3N?[a]		High. Estimated mortality of 270,000–360,000 in Europe	Perhaps H3N8 virus
1918	39	H1N1	Spanish, swine	548,000	Possible reassortant between a preexisting human H1 virus lineage and an avian virus[b]
1957	11	H2N2	Asian	86,000	Avian-human reassortant virus
1968	Ongoing	H3N2	Hong Kong	34,000	Avian-human reassortant virus
1976	<1	H1N1	Swine	1 death	Outbreak limited to one U.S. military base
1977	31	H1N1	Russian	Negligible	Reappearance of earlier circulating virus
2009	Ongoing	H1N1	Swine, A(H1N1)pdm09	12,500[c]	Swine-origin reassortant virus

[a]The subtype designation of this virus is uncertain but is proposed on the basis of retrospective serologic studies of elderly adults who were living at the times of the pandemic (77).
[b]Earlier work postulated that the origin of the 1918 H1N1 virus represented an introduction of all eight genes from an avian ancestor or a reassortant involving progenitor viruses circulating in humans and swine (2, 4, 74).
[c]First year of circulation.

genetic reassortment in cells dually infected by a human and an animal virus (Fig. 5) or possibly by direct interspecies transmission (33). When such viruses are introduced into a susceptible population, they cause zoonotic infections, and if efficiently transmitted from person to person, can lead to pandemics.

Pandemic Influenza

Over the past 300 years, at least seven pandemics of influenza have probably occurred, including three well-characterized ones in the 20th century and one starting in 2009 (69). These have appeared at irregular intervals and been notable for efficient transmission, high illness rates in all susceptible age groups, particularly in young persons, and usually for significant increases in mortality rates (Table 4). Earlier pandemics have spread globally over 6- to 9-month periods at the pace of human traffic and often irrespective of season. However, the 2009 pandemic H1N1 virus [A(H1N1)pdm09] disseminated much more rapidly, in part because of air travel patterns (70, 493). The estimated median reproduction (R) values for the 1918 (1.80), 1957 (1.65), and 1968 (1.80) pandemics are somewhat higher than those for the 2009 pandemic (1.46) or seasonal epidemics (1.28) (71). Second and sometimes third waves of infection separated by several months may occur. As observed with the 2009 pandemic, the impact of subsequent waves can sometimes exceed that observed during the initial one (69).

Cumulative illness rates have often exceeded 50% in the general population during successive waves of a pandemic. During its first year of circulation, the A(H1N1)pdm09 virus was estimated to have caused approximately 60.8 million cases, 274,304 hospitalizations, and 12,469 deaths in the United States (72), as well as an estimated 151,700 to 574,900 respiratory and cardiovascular deaths worldwide, over half of which occurred in southeast Asia and Africa (73). Marked age-related differences in impact often occur with pandemics. Older adults usually experience lower illness frequency, likely related to prior experience with other influenza viruses and perhaps less frequent exposure, but the case fatality ratios are usually highest in the very young and elderly. During the 2009 pandemic mortality rates in the United States increased 8.5-fold in those 0 to 17 years old and 12.5-fold in those 18 to 64 years old compared to seasonal influenza, but decreased 5.3-fold in those 65 years and older (72). The 1918 pandemic also caused an increase in mortality in young adults, perhaps because an H1 virus circulated in the human population before 1889 and resulted in partial protection against the 1918 virus in the older segment of the population. In adults aged approximately 20 to 40 years a possible adverse effect of childhood priming with a heterosubtypic influenza A virus has been postulated (74). During the 1918 pandemic, >30-fold differences in population mortality occurred across countries and about 4-fold differences occurred within countries, including the United States (75). In the era encompassing virus isolation, pandemics in 1957 and to a lesser extent in 1968 also caused increases in mortality compared to interpandemic periods (Table 4). In each of the recent pandemics, large portions of the excess influenza-related deaths (estimated >99% in 1918, 36% in 1957, 48% in 1968, 87% in 2009) occurred in those aged <65 years in the United States, followed by smaller proportions over the subsequent decade (72, 76). In contrast, disease due to the reappearance of an A(H1N1) subtype virus in 1977, after a 20-year hiatus since circulation of essentially the same strain, principally affected those younger than 25 years and caused negligible excess mortality.

Serologic studies with elderly individuals suggest that viruses with HA of the H3 subtype caused pandemic disease in the late 19th century (Table 4) (77). The origin of these pandemic strains and the mechanisms for apparent recycling of strains in the human population remain unresolved issues. Explanations include dormancy in a frozen state, which may have been the case for the 1977 A(H1N1) strain, interspecies transmission and adaptation of an animal influenza virus to become infectious and pathogenic for humans, and, more commonly, genetic reassortment between animal and human influenza viruses. Analysis of the 1957 pandemic strain found that this A(H2N2) subtype virus resulted from acquisition of three new gene segments (HA, NA, and PB1) of avian origin by the previously circulating human A(H1N1) strain. Similarly, the 1968 pandemic A(H3N2) virus acquired two new genes (HA and PB1) from an avian virus closely related to viruses isolated from ducks in Asia in 1963 (69) (Fig. 5).

Changes in the receptor specificity of the avian influenza virus HA appear to be one key factor in generation of viruses

that are capable of sustained human transmission, and the HAs of all pandemic viruses characterized to date bind to human-like *a*2,6-sialylated glycans. One or two amino acid changes can alter receptor binding patterns of the 1918 virus HA and reduce transmissibility among ferrets without altering replication or lethality, and one 1918 virus HA from a fatal case shows dual *a*2,3 and *a*2,6 binding (78). Avian A(H5N1) viruses transmit inefficiently or not at all among ferrets, despite several H5 HAs showing mutations associated with dual *a*2,3 and *a*2,6 binding (79). Although the predictive value of the ferret model for human transmission is uncertain, several A(H5N1) viruses engineered to bind preferentially to *a*2,6 receptors and selected by serial ferret passage show airborne transmission in ferrets (80). Changes in multiple genes are likely necessary to generate a pandemic virus.

Although avian viruses usually cannot directly infect humans, swine can be infected by both human and avian viruses and may serve as an intermediate host needed for reassortment of these viruses, the so-called ``mixing vessel," or as the species in which avian viruses can adapt to a mammalian host. Avian-human reassortment and avian-like influenza A viruses with limited zoonotic spread to humans have been identified in swine (81). Of note, Eurasian (contributing NA, M genes) and North American (HA, NP, NS) lineages of swine A(H1N1) viruses, the latter having undergone earlier reassortment events to acquire both avian (PA, PB2) and human (PB1) gene segments, reassorted to form the A(H1N1)pdm09 virus (82). This virus was reintroduced from humans into swine in which new reassortants have emerged (75), including the swine A(H3N2) variant virus that caused a large zoonotic outbreak in 2012 (see below) after having acquired the Eurasian M gene from A(H1N1) pdm09 (83). The origin of the genes of the 1918 pandemic strain remains unknown, but it is likely that some of the genes were derived from avian influenza viruses (3, 4, 74).

The ecology and interspecies transmissions of influenza viruses are complex. At least two of the 20th-century pandemic strains likely emerged from China, whereas the A(H1N1)pdm09 virus first appeared in North America. The concept of China and Southeast Asia as the site of origin or epicenter of new pandemic viruses has been supported by the presence of high concentrations and cohabitation of domestic poultry (especially ducks), swine, and humans. The avian reservoir provides a mechanism for maintaining and reassorting virus genes, as well as a source for the introduction of novel subtypes into humans (Table 3). For example, avian viruses, which contain an antigenically conserved H2 HA similar to that of the 1957 pandemic strain, continue to circulate in birds and have been identified in swine (84).

Epidemic Influenza

Seasonality

Influenza activity of varying intensity occurs annually in temperate areas, most often during the winter or early spring months (Fig. 8). The distinct seasonality of epidemic influenza appears to be related to reintroduction of virus each season, behavioral factors influencing exposure (e.g., school attendance and indoor crowding), factors affecting viral survival in the environment (e.g., low temperature and humidity), and possibly host determinants influenced by seasonal changes (41, 85). Low temperature and low relative humidity enhance transmission in the guinea pig model, thus confirming one explanation for the wintertime seasonality of influenza A and B viruses (86–89). A(H3N2) epidemics in temperate regions are seeded from temporally overlapping epidemics in East-Southeast Asia or other sites and not from persistent circulation of virus in temperate areas during the summer months (60, 64, 90, 498).

The onset of influenza activity ranges from October to April in the northern hemisphere but usually peaks between December and March (between May and August in the southern hemisphere) (Fig. 8). More than one epidemic annually occurs in some tropical countries, and year-round activity may be present in the tropics (91). Summertime

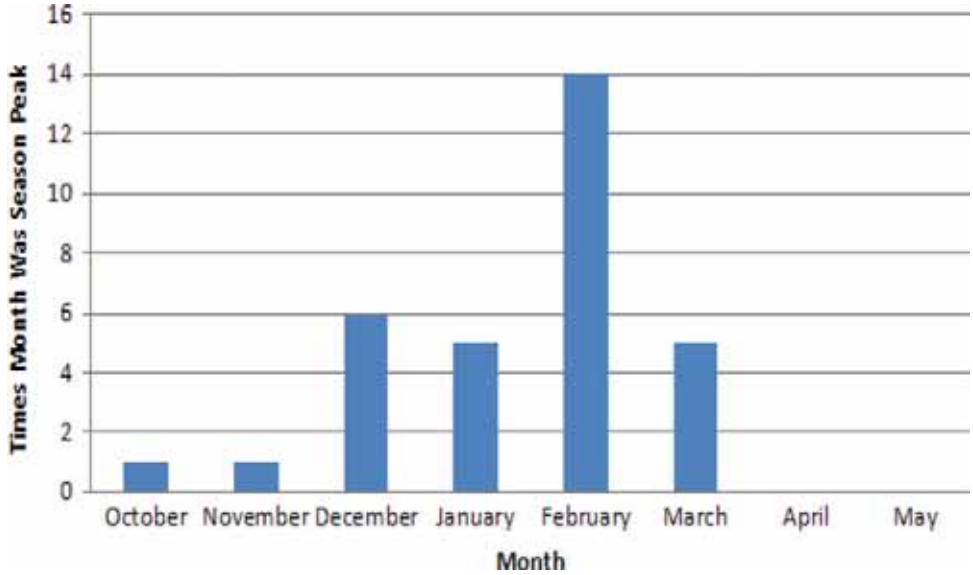

FIGURE 8 Peak month of influenza activity, USA, 1982–2014. During 2008–2009, influenza activity peaked twice because of the 2009 H1N1 pandemic. Activity in the United States peaked once in February due to seasonal influenza activity and then again in June, with the first wave of A(H1N1)pdm09 virus, followed by a second, larger peak of A(H1N1)pdm09 virus activity occurring in October, the peak of the 2009–2010 season. (Figure reproduced with permission from Influenza Division, US CDC.)

outbreaks may occur on cruise ships and among large groups of land-based travelers, especially when new antigenic variants are introduced from elsewhere. Consequently, an influenza diagnosis should be considered outside the influenza season in those with febrile respiratory illness who have recently traveled in the tropics, in the southern hemisphere, or with international groups (92).

In a given community or region, outbreaks due to a particular virus usually peak in about 3 weeks and are of short duration (6 to 10 weeks). Successive or overlapping waves of infection by different influenza A subtypes and influenza B virus may cause prolonged influenza activity, such that the overall epidemic influenza lasts 3 to 5 months within countries (91). Influenza B viruses cause widespread epidemics every 3 to 4 years, but co-circulation of two or three influenza viruses (H3, H1, and B lineages) may occur within a single season. The isolation of an antigenically drifted virus late in the spring months during a limited outbreak (herald wave) may foreshadow an epidemic in the next season.

Community Impact

Seasonal influenza occurs globally with variable annual attack rates higher in children (estimated 20 to 30%) than adults (estimated 5 to 10%) (494, 500, 501). The overall incidence of seasonal influenza is highest in school-age children and adolescents and declines with increasing age, such that attack rates are about four-fold lower in people over age 60. School-age children play a central role in the dissemination of influenza in the community and spread virus to their younger siblings and parents in the household. Most secondary cases in households occur within 1 week of symptom onset in the index case (57). Increased school absenteeism is an early epidemiological marker of epidemic influenza, and early school closings may blunt outbreaks and even pandemic waves (93, 94). Epidemic attack rates of 60 to 75% have been described to occur in schoolchildren. Worldwide in children < 5 years old, there were an estimated 90 million (95% CI: 49–162 million) new cases of seasonal influenza and 20 million (95% CI: 13–32 million) cases of influenza-associated acute lower respiratory infections during 2008 (95).

Epidemics are also marked by increases in visits to primary care physicians and emergency rooms for febrile respiratory illness, workplace absenteeism, and subsequently hospitalizations for pneumonia and underlying conditions. Influenza is the single most common cause of medically attended acute respiratory illness. Up to one-half of influenza A(H3N2) subtype or B illnesses lead to physician contact. Approximately 5 to 15% of teens or adults and 25% of children < 5 years old seek medical care for acute respiratory disease during epidemics (512). Influenza virus has been associated with 15 to 25% of all respiratory illnesses seen by physicians and up to 40% of those seen in patients over the age of 15 years.

The impact of influenza varies considerably with age, underlying medical comorbidities, and the epidemic strain (96). In the United States, increases in pneumonia and influenza hospitalizations range from 16,000 to more than 220,000 per epidemic, less than one-half of which involve adults 65 years of age or older. Admissions for exacerbations of chronic respiratory diseases and cardiovascular disease, particularly congestive heart failure, also increase. The peak of hospitalizations lags about 1 week after the peak of influenza activity. Hospitalization rates for all influenza-related complications are about 1 to 3 per 1,000 persons. During 15 years to the 2007–08 season, the estimated seasonal influenza-related hospitalizations for respiratory and circulatory disease averaged $63.5/10^5$ person-years but varied about 3-fold across seasons and were highest among persons aged ≥ 65 years ($309/10^5$) and those aged < 1 year ($151/10^5$) in the United States (97). Among children, hospitalization rates are highest in those < 6 months of age and decline through 6 years of age. A(H3N2) subtype epidemics are associated with two- to threefold increases in pneumonia rates and two- to fivefold increases in hospitalization rates for adults with high-risk medical conditions. Diabetic persons experience increased hospitalizations and mortality due to ketoacidosis and pneumonia during influenza epidemics. However, most adults, but less than one-half of children, requiring hospitalization for respiratory disease have a previously recognized underlying medical condition (98). Contemporary strains of A(H3N2) subtype viruses typically cause the highest hospitalization and mortality rates (Fig. 9).

Mortality

The overall case fatality of seasonal influenza is low, generally 0.01% or less, but epidemics are often associated with mortality rates well in excess of those observed during comparable time periods in the absence of influenza outbreaks. In the United States annual influenza-associated mortality is estimated to have ranged from 3,349 to 48,614 deaths (1.4 to $16.7/10^5$ persons) between 1976–2007 (99) and from 4,915 to 27,174 during the 3 seasons from 2010–2013 (100). Mortality rates are highest in infants, the elderly, and those with underlying cardiopulmonary disease. Globally, during 2008, an estimated 28,000 to 111,500 deaths occurred in children < 5 years of age, almost all of which occurred in developing countries (95). For seasonal influenza, about 90% of deaths in developed countries occur in those ≥ 65 years of age, and the mortality rates in those over 65 years old are more than 50-fold higher than in younger persons (72) and are especially high in those aged 85 years and older.

Most seasonal influenza mortality is attributable to lower respiratory complications or cardiovascular disease (503). Pneumonia and influenza deaths account for only about one-quarter of all excess deaths, but they serve as important markers of influenza activity (Fig. 9). Up to two-thirds of the excess deaths are related to cardiovascular disease. Excess mortality due to pneumonia and influenza-related deaths in the United States occurs particularly in association with A (H3N2) subtype epidemics. Increases in mortality rates generally lag several weeks behind the peak of influenza activity and persist for approximately 6 weeks afterwards.

Influenza C

Influenza C virus infection is worldwide in distribution and generally nonseasonal but may cause prolonged community outbreaks (101, 102). Symptomatic infections usually occur between 1 and 4 years of age, but influenza C virus usually represents < 1% of viruses from children with respiratory illness (101, 102). Most children ≥ 6 years and adults are seropositive. Influenza C virus has been associated with outbreaks in closed populations, including those in children's homes and the military.

Nosocomial Infections

Influenza A and, less often, B viruses are important causes of nosocomial outbreaks involving hospital wards, intensive care units, nursing homes, and other semi-closed populations (53, 103). Because of the short incubation period of influenza and its efficient transmission, rapid dissemination of virus can lead to explosive outbreaks in such populations.

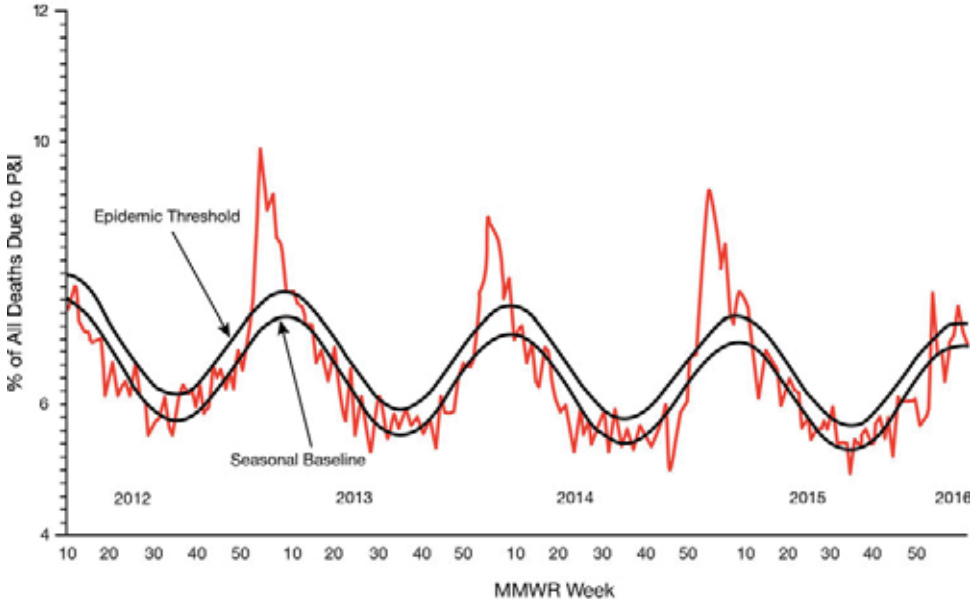

FIGURE 9 Pneumonia and influenza (P&I) deaths presented as a percentage of total deaths in the United States, 2011 to 2016. Total deaths and those attributed to pneumonia and influenza deaths are reported weekly from 122 cities in the United States to the CDC. Excess mortality above the epidemic threshold has been seen particularly in association with influenza A (H3N2) subtype activity. A(H3N2) viruses predominated nationally during the 2012–13 and 2014–15 seasons, and there was a mismatch with the vaccine strain during the latter season. However, during the 2013–14 season, A(H1N1)pdm09 viruses predominated and increased overall mortality, and higher rates of hospitalization among adults aged 50 to 64 years occurred. Figure reproduced with permission from the Influenza Division, US CDC.

Outbreaks are typically brief, lasting from 1 to 3 weeks. Clinical attack rates among groups of institutionalized adults range from 10 to 60%, with substantial ward-to-ward variation. Lower respiratory tract complications develop in one-third or more of affected adults, and mortality rates range widely (0 to 30%). Healthcare workers (HCWs) are often involved during the course of both community and hospital outbreaks of influenza. The incidence of influenza infection among unvaccinated HCWs has been estimated at 16 to 22% per season (104). During outbreaks secondary HCW infections are common (20 to 50% attack rates) and can lead to absenteeism and disruption of services. Infected workers can initiate or perpetuate outbreaks by transmitting infection to susceptible patients, and healthcare-provider immunization has been associated with reduced influenza-associated illness and mortality in chronic-care facilities (105).

Nursing home residents are disproportionately impacted by influenza because of higher frequencies of underlying disease, decreased vaccine responsiveness, and greater likelihood of influenza exposure, due to residence in semi-closed facilities, compared to elderly persons in the community. Nursing home outbreaks are more likely in larger homes and in those with lower immunization rates (106), although outbreaks have occurred despite immunization rates exceeding 90% and sometimes out of season during the summer months. Nosocomial influenza should be considered in hospitalized or nursing home patients with unexplained febrile illness or respiratory deterioration, especially during periods of influenza activity in the community or if multiple cases occur.

Zoonotic Infections

Animal influenza viruses cause occasional zoonotic infections, but such events are usually associated with inefficient human-to-human spread, although they create the conditions for the potential emergence of an animal-human reassortant virus. Ongoing surveillance in animal populations and their human contacts is essential to look for new variants that may pose threats and warrant the development of candidate vaccines (107).

Swine Influenza Viruses

Swine-origin influenza A viruses that cause zoonotic infections are now termed variant (v) influenza viruses and include H1N1v, H1N2v, and H3N2v subtypes that have caused illness in humans uncommonly cause zoonotic infection. Human influenza viruses also transmit to swine and may contribute to generating new variants (108). Transmission of A(H1N1) subtype virus from swine has been frequently implicated, including the Fort Dix outbreak in 1976 and sporadic fatal pneumonic disease, particularly in pregnant or immunocompromised persons (81). Influenza virus is readily detectable in the air and environment of live animal markets and production facilities containing infected swine, and swine workers and their close contacts have increased risk of infection by such viruses (109, 110). During the summer of 2012 H3N2v outbreaks across 10 U.S. states caused 306 proven cases including 16 hospitalizations and 1 death; more than 90% of those infected were children and reported agricultural fair attendance and/or contact with swine, although some limited human-to-human transmission likely occurred (111). Of note, transmission of influenza viruses to humans can occur from healthy-appearing swine.

A low prevalence of antibodies to influenza D virus has been found in humans (112), but it is unclear whether such viruses may cause disease in humans. Detection of a novel A(H2N3) subtype virus in swine has not been associated with recognized human infection (84).

Avian Influenza Viruses

Serologic evidence of infection by multiple subtypes of avian viruses is present in Chinese agricultural workers, and

experimental human infection has developed after intranasal exposure to H4, H6, and H10 subtype viruses (113). Purulent conjunctivitis due to A(H7N7) subtype influenza virus has followed exposure to infected seals or laboratory materials (Table 3). In 2003, a large outbreak of highly pathogenic avian influenza (HPAI) A(H7N7) virus in poultry in Holland and Belgium was associated with many human illnesses, principally conjunctivitis but also influenza-like illness and one fatal pneumonia in a veterinarian, as well as evidence of human-to-human transmission (114). Sporadic human infections by other H7 subtype viruses, sometimes without apparent symptoms and detected serologically, have been reported in multiple countries. Avian A(H9N2) subtype viruses continue to circulate widely in poultry populations in parts of Asia, Africa, and the Middle East. While these viruses have caused few recognized human infections (Table 3), they have been the source of key internal genes implicated in the emergence of other avian viruses causing zoonotic infections including both A(H5N1) and A(H7N9) viruses (115, 116). Of great concern have been sporadic cases of avian influenza infections, particularly major outbreaks of avian A(H5N1) and A(H7N9) viruses (Table 3). Increased poultry infection-associated risk to humans occurs during the winter months when seasonal influenza viruses are circulating, thus creating the potential for dual infections (117).

H5N1 Virus

In 1997, direct interspecies transmission of a highly pathogenic avian influenza (HPAI) A(H5N1)-subtype virus led to 18 illnesses and six deaths in Hong Kong (Table 3) (118). This experience established that avian influenza viruses could cause human infections without adaptation in an intermediate host and despite differences in receptor specificity. The virus possessed a polybasic amino acid HA cleavage site characteristic of HPAI viruses and was uniquely lethal in animals without adaptation (119). The virulent A(H5N1) subtype appeared to arise as a reassortant of avian influenza viruses, likely including a goose A(H5N1) subtype virus and possibly a quail A(H9N2) subtype virus, which provided multiple internal genes. Subsequent reassortment events and genetic changes led to further variants causing fatalities in one family visiting Fujian Province in 2003, and then major poultry outbreaks and associated human cases in Thailand, Vietnam, and Cambodia starting in 2003 to 2004, followed by spread across Asia to Europe, the Middle East, and Africa (35). Poultry raising and trade practices, live-poultry markets, duck abundance, and rice-cropping intensity are contributory to the persistence of the virus, which is currently entrenched in poultry populations of multiple countries in Asia and Africa (notably China, Egypt, Indonesia, parts of India and Bangladesh, the Greater Mekong subregion, and, likely, parts of West Africa). Sporadic cases continue to occur, and a major outbreak affecting 165 persons, almost all with history of poultry contact, caused a 35% case-fatality rate in Egypt during the winter months of 2014–2015 (120). The HA gene has continued to evolve, with 10 first-order clades recognized in poultry, four of which (clades 0, 1, 2, and 7) have caused fatal human illness (121), and multiple higher order ones (122).

The epizootic in birds has resulted in loss through illness or culling of hundreds of millions of birds, predominantly chickens and ducks, but has resulted in relatively few human cases to date, despite numerous exposures (Table 3). Episodes of nonsustained person-to-person transmission to household and healthcare contacts have been recognized uncommonly; subclinical human infections have also been uncommon to date (121). One explanation for the rare event of a chicken-to-human transmission is that only large amounts of virus lead to infection (and disease) in humans, but the host factors that might contribute to susceptibility are poorly understood. Of note, the combination of relatively few substitutions in the HA, including change in HA binding preference from a2,3-linked to a2,6-linked sialic acid receptors, and in the polymerase complex, confer airborne transmissibility of A(H5N1) in ferrets, a model thought by some investigators to be predictive of human transmissibility (80, 123, 124). Some of the required changes already exist commonly in field isolates (125). However, the fact remains that the virus-host interactions leading to effective transmission of avian viruses in humans remain undetermined.

Since early 2014, rapid spread of HPAI H5Nx reassortant viruses, possessing the genetic clade 2.3.4.4 HA, has occurred among wild and domestic birds leading to poultry outbreaks in Asia, Europe, and, for the first time, in North America (107). A HPAI A(H5N8) virus, first detected in January 2014 in South Korea, spread within the year to North America leading to emergence of reassortant A(H5N2) and A(H5N1) viruses that caused loss of approximately 40 million chickens and turkeys in the United States in 2015, although there were no recognized human cases (126). These viruses are less virulent in mouse and ferret models than an Asian lineage A(H5N1) virus and are not transmitted by direct contact in ferrets (127). Outbreaks of A(H5N1) and A(H5N2) viruses in poultry have also occurred in European countries. Their spread may have occurred through infections in wild birds and migratory bird dispersal, activities associated with poultry production, and possibly, in some countries, by illegal poultry transports.

H7N9 Virus

Avian A(H7N9) virus infection was first detected in fatal human cases in March 2013 (115) and has subsequently caused at least 770 cases in China, with a case fatality exceeding 30% in hospitalized (128). This novel virus arose from sequential reassortments in domestic poultry involving genes from A(H9N2) donor viruses, as well as from H7 and N9 precursors from wild birds. Although composed of avian influenza genes, the virus contains some markers of mammalian adaptation, including the ability of some isolates to be transmitted by airborne route in ferrets (129, 130). Lacking a polybasic amino acid cleavage site in HA, the virus does not cause overt disease in infected birds, which makes recognition and control difficult. The virus is now widely endemic in poultry markets in China. Most human cases have resulted from exposure to live birds or live bird markets prior to onset of disease, and large waves of human infections occurring during the winter months have ceased in temporal association with closures of live bird markets (131, 132). Family clusters and several instances of nosocomial transmission to unrelated healthcare workers have occurred (133–135). In contrast to A(H5N1) and A(H1N1)pdm09 infections, older males with comorbidities have been disproportionately affected by severe A(H7N9) disease (136, 137). Many milder infections have likely taken place (134), and subclinical infections, specifically in poultry workers, have been detected (138), although serologic studies indicate that most persons in the community lack specific antibody and hence are susceptible.

PATHOGENESIS

Viral Replication

The initial site of infection is the respiratory tract mucosa (12) and is mediated by HA binding to sialylated glycans on epithelial cells. The α2,6-linked sialic acid receptors preferred by human viruses are present in both the upper and lower respiratory tract, particularly tracheobronchial epithelium and type 1 pneumocytes, whereas the α2,3-linked receptors preferred by avian viruses are present in the distal bronchioles, type 2 pneumocytes, and alveolar macrophages, as well as conjunctival mucosa (139). These distribution patterns may explain in part the high frequency of tracheobronchitis in seasonal influenza, conjunctivitis in avian A(H7) infections, and viral pneumonia but the relative paucity of upper respiratory manifestations in avian A(H5N1) and A(H7N9) infections.

In experimentally infected volunteers, virus shedding begins about 1 day before onset of illness, and titers in nasal washings peak within several days at 10^4 to 10^7 50% tissue culture-infectious doses ($TCID_{50}$) per ml (140). In uncomplicated influenza A virus infections, viral RNA titers in upper respiratory secretions are often detectable 1 to 2 days before symptom onset, peak on the first 1 to 2 days of clinical illness, and decrease gradually to undetectable levels by day 6 to 7, generally matching the dynamics of clinical illness; influenza B virus RNA titers may be more sustained (48). The duration of infectious virus detection in upper respiratory secretions is generally 3 to 6 days during uncomplicated influenza in adults and older children. Viral loads determined by molecular assays for viral RNA are higher and more prolonged, generally by 2 to 3 days (141). Fever and the severity of illness correlate temporally with the quantity of virus detectable in respiratory secretions. Hospitalized adults may have detectable infectious virus or viral RNA in upper respiratory samples for a week or longer after illness onset; comorbidities and systemic corticosteroid use are associated with slower viral clearance (142, 143). Nasopharyngeal infectious virus levels correlate with viral RNA loads in hospitalized adults but decline more rapidly, and are often negative, when RNA detectability persists (144). Longer periods of shedding (1 to 3 weeks) are commonly seen in infants and children, in critically ill patients, and in those experiencing severe zoonotic infections (121, 141, 145–148). In avian A(H5N1) and A(H7N9) disease, viral levels are higher in lower respiratory than in upper respiratory tract specimens and higher in the throat than in the nose. Prolonged virus shedding (weeks to months) has been described to occur in patients with immunodeficiency, including advanced human immunodeficiency virus (HIV) disease (149, 150).

Recovery of infectious influenza virus from blood (viremia) or extrapulmonary tissues has been documented rarely in seasonal influenza. Virus has been recovered from the heart, liver, spleen, kidneys, adrenals, muscles, and meninges, and, in children with encephalopathy, viral RNA has been detected sometimes in cerebrospinal fluid (CSF). Detection of viral RNA in blood (RNAemia), found in as many as 10% of those hospitalized with A(H1N1)pdm09 illness, has been associated with more severe clinical presentation and higher mortality (136, 151). In HSCT-transplant patients with influenza, the detection of viral RNA in the blood is associated with disease progression and mortality (152). Viral RNA positivity in stool may be associated with gastrointestinal symptoms (153, 154). Viremia, gastrointestinal infection, and extrapulmonary dissemination, sometimes including the central nervous system (CNS), occur in some avian A(H5N1)-infected patients, and detection of viral RNA in blood or feces is associated with a worse prognosis (121, 147, 155).

Pathological Changes

In apparently uncomplicated influenza, bronchial histopathology shows degeneration of respiratory epithelial cells with loss of ciliated tufts and desquamation, pseudometaplastic changes of the epithelium, and edema, hyperemia, and mononuclear cell infiltrates in the lamina propria (156, 504). In fatal influenza viral pneumonia, the gross pathological findings include hemorrhagic, airless lungs and severe tracheobronchitis (157). The pathological features include necrotizing tracheobronchitis and bronchiolitis with loss of ciliated epithelium, fibrin exudation, and inflammatory cell infiltration; diffuse alveolar damage with hyaline membrane formation and intra-alveolar and intrabronchiolar hemorrhage; and interstitial edema, hemorrhage, and mononuclear cell infiltration (Fig. 10A-C) (156). Later changes include lymphohistiocytic alveolitis, metaplastic epithelial regeneration, and sometimes extensive fibrosis (Fig. 10D). Influenza virus antigens (Fig. 10C) or RNA have been demonstrated in virtually all cell types of the respiratory tract, including type 1 and 2 pneumocytes, and virus has been recovered from pulmonary tissues as late as 3 weeks after illness onset in fatal cases and sometimes later in those treated with corticosteroids (155). Similar pulmonary changes occur in severe viral pneumonias caused by diverse influenza viruses, including the 1918 pandemic virus, A(H1N1)pdm09, A(H5N1), and A(H7N9) viruses (157–159). When bacterial superinfection develops, lung pathology is dominated by an influx of polymorphonuclear leukocytes (Fig. 10E).

Both direct cytopathic effects (CPE) and virally induced apoptosis contribute to the pathological changes. Exuberant local and systemic cytokine and chemokine responses (145, 148, 160) have been postulated to cause inflammation and cellular infiltration in the lungs and other organs. In the mouse lung, a subpopulation of lung epithelial cells, club cells (formerly Clara cells), survive for weeks after infection producing proinflammatory mediators, which initially contribute to an antiviral response but, after prolonged activation, enhance immunopathology (161, 162). The severity of pneumonitis relates to cell-mediated immune responses and can be enhanced by transfer of certain types of T lymphocytes in murine models, but the extent to which such immunopathological host responses contribute to human disease remains uncertain. Lymphocytopenia in lethal human influenza appears to be associated with a lung T lymphocytosis, although not necessarily increased cytokine elaboration (163). Of note, nonneutralizing, cross-reacting antibodies, that lead to immune complex formation and complement deposition in the lung, are postulated to be a major pathogenetic mechanism leading to severe pulmonary disease, and, in contrast to seasonal influenza, extensive C4d deposition in bronchioles has been demonstrated in fatal A(H1N1)pdm09 and 1957 pandemic influenza cases (163).

Patients with fatal cases of influenza often show pathological changes in other organs. Diffuse congestion and generalized swelling of the brain and myocardial inflammation with edema, interstitial hemorrhage, myocyte necrosis, and lymphocytic infiltration have been found in one-third or more of autopsies. Pathologic evidence of acute myocardial injury has been found in a majority of fatal influenza B cases in children (164). The few autopsies of patients with fatal A(H5N1) and A(H7N9) have also shown lymphocyte

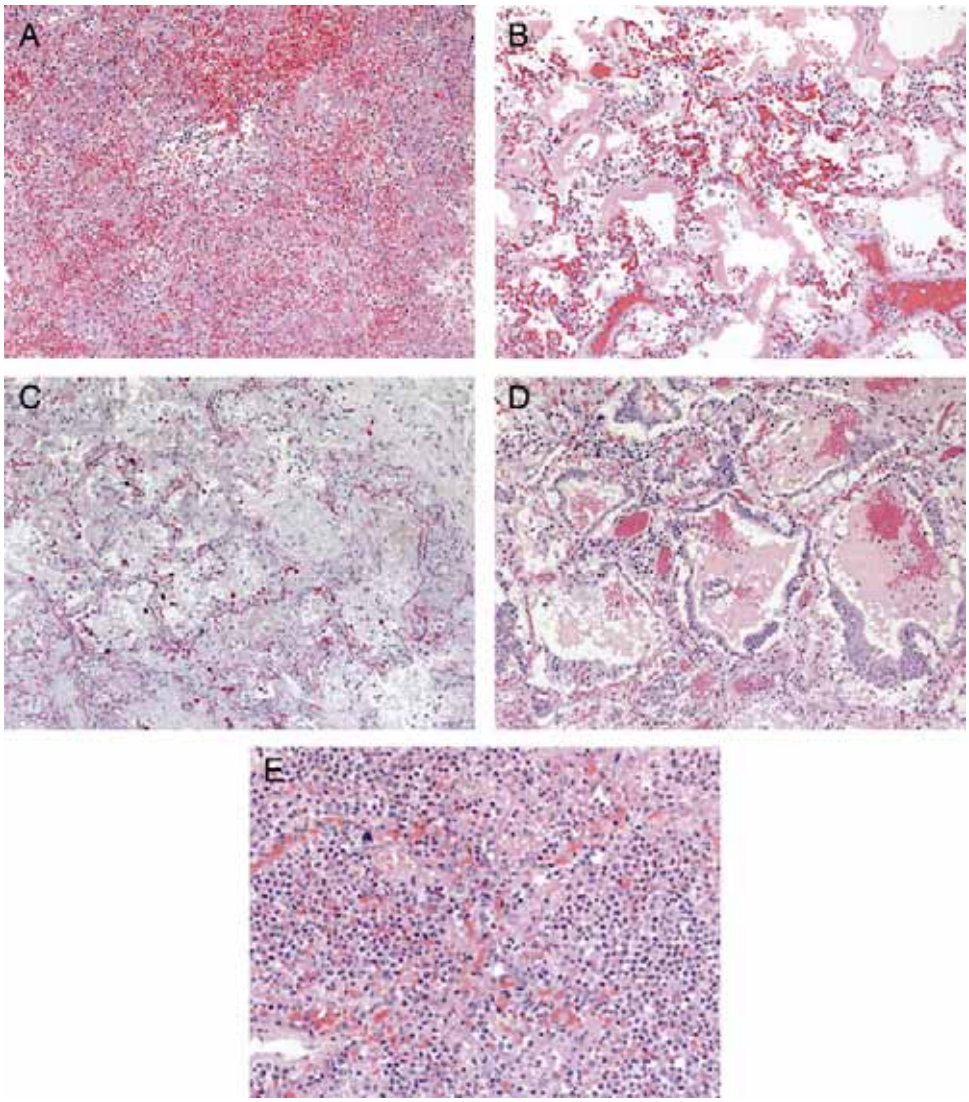

FIGURE 10 Photomicrographs of lung tissue from patients dying with primary viral (A-D) or secondary bacterial (E) pneumonia following influenza A(H1N1)pdm09 infection. Unless otherwise indicated, histologic sections were stained with hematoxylin and eosin. (A) Diffuse alveolar damage and alveolar hemorrhage with erythrocytes and exudate filling alveoli; (B) extensive hyaline membrane formation; (C) widespread influenza A nucleoprotein antigen staining by immunohistochemistry; (D) early regenerative phase with type II pneumocyte hyperplasia and alveolar exudates; (E) polymorphonuclear leukocytes (PMNs) filling alveolar spaces in *Streptococcus pyogenes* suprainfection. Photomicrographs kindly provided by Dr. Sherif Zaki, US Centers for Disease Control and Prevention, Atlanta, Georgia; republished with permission from reference 158.

depletion, hemophagocytosis, hypoxic changes in liver and kidney, and hepatitis, consistent with observed changes in clinical laboratory values (157, 159).

Pathogenesis of Symptoms

Direct viral involvement of the upper and lower respiratory tract accounts for much of the illness associated with influenza, particularly the high frequencies of cough and tracheal irritation, coryza, and sore throat. Even in apparently uncomplicated influenza, bronchoscopy shows tracheobronchial inflammation, and pulmonary function abnormalities persist for weeks to months after infection (223, 224, 504). Pulmonary function studies have detected restrictive and obstructive ventilatory defects, abnormal gas exchange with increased alveolar-arterial oxygen gradients, decreased carbon monoxide diffusing capacity, and airway hyperreactivity.

The cause of the marked constitutional symptoms during influenza relates, in large measure, to elaboration of proinflammatory cytokines and chemokines. During experimental human influenza, nasal lavage levels of IFN-α, IFN-γ, interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), IL-8, IL-1b, IL-10, MCP-10, and MIP-1α and -1β and blood levels of IL-6 and TNF-α levels occur; early increases in nasal IFN-α, IFN-γ, and IL-6 correlate with viral titers and illness measures (140). Interferon levels increase in respiratory secretions and blood during uncomplicated human influenza. Interferon concentrations peak about 1 day after the peak of virus shedding and fall in temporal association with

decreases in viral replication. In uncomplicated influenza, levels of IL-6, IFN-α, IFN-γ, TNF-α, and IL-10 in the nose and blood are increased early; IL-6 levels correlate with illness measures, and nasal IFN-γ levels correlate with decreases in viral titers (165).

Hypercytokinemia occurs in patients hospitalized with serious human influenza, in whom elevated plasma IL-6 levels correlate with prolonged hospitalization (166), and is especially marked in A(H5N1) patients, in whom the highest cytokine levels are found in those with fatal illness (147). High plasma levels of IL-6, IL-8, CCL-2 (monocyte chemoattractant protein-1), and soluble TNF receptor-1 occur in severe A(H1N1)pdm09 pneumonia and complicated seasonal influenza and correlate with the extent and progression of pneumonia (148, 160). In A(H7N9) influenza virus infections, high plasma concentrations of IL-6, IL-8, IL-10, and MIP-1β are also predictive of poor or fatal outcome (137). Matched bronchoalveolar lavage (BAL) samples show up to 1,000-fold greater cytokine/chemokine levels relative to plasma, indicative of local elaboration in the lung.

The mechanisms of cytokine induction are incompletely defined, but the extent of viral replication is an important variable. Upper respiratory tract viral loads in patients hospitalized with either seasonal influenza or A(H5N1) infection correlate with plasma cytokine and chemokine levels (147, 166). Viral virulence factors also play a role (see below). For example, human isolates of A(H7N9) virus replicate more efficiently in *ex vivo* cultures of human bronchus and lung than A(H5N1), but A(H5N1) virus are more potent inducers of pro-inflammatory cytokines than A (H7N9) or A(H1N1)pdm09 viruses (167). Influenza HA activates cellular transcription factor-NF-κB binding and transactivation of target genes encoding certain cytokines, cellular adhesion molecules, and other acute-phase proteins. Viral NA can also stimulate TNF and IL-1 production by monocytes and activate latent-transforming growth factor *β*. Virus-associated double-stranded RNA, interacting with Toll-like receptors 3 and 7 and with the helicase retinoic acid-inducible gene (RIG-I), appear to play an important role in initiating acute-phase responses and inducing cytokine elaboration during acute influenza (168).

Viral Pathogenicity

Virulence is a multigenic characteristic that does not relate to a specific subtype and varies within subtype. Viral genetic features that increase replication, especially in the distal airways and lungs, inflammatory responses, or pathogenicity contribute to disease severity. The H1N1 pandemic of 1918 was especially severe, likely related to the greater pneumotropism of the virus, absence of effective herd immunity, and the consequences of secondary bacterial infections (169). Unlike seasonal viruses, the 1918 virus causes a lethal pneumonia with sustained viral replication in experimentally infected macaques and a dysregulated immune response characterized by deficient interferon responses early and prolonged proinflammatory responses (170). The polymerase genes from the 1918 influenza virus are required for full pathogenicity in animals (3, 4). Some influenza A viruses like 1918 pandemic virus and A(H5N1) express full-length PB1-F2 that contributes to cytotoxicity and inflammatory responses in the lungs and airways, and one mutation in PB1-F2 (Asp66Ser) is associated with increased viral replication (171, 172).

Requirements for airborne transmissibility in ferrets appear to include HA acid stability, with lower pH needed for HA cleavage activation, binding capacity to α-2,6 sialic acid receptors, and efficient polymerase activity at 33°C, the temperature of mammalian upper airways (173). The extent of HA glycosylation status affects lung tropism, and viruses possessing a poorly glycosylated HA, as well as the ability to bind both α2,3-and α2,6-linked sialic acid-bearing receptors, are able to penetrate deep into the lungs and increase the risk of bacterial co-infections (174). Pathogenicity and cell tropism of influenza viruses also relate in part to the HA cleavability by particular host cell enzymes (33). Serine proteases, presumably derived from host epithelial cells, cleave the HA precursor molecule into HA1 and HA2 to render human influenza viruses infectious. Protease activity present in human nasal secretions, common house dust mites, and proteases produced by some bacteria, including *Staphylococcus aureus*, can enhance viral replication. Certain bacterial enzymes like streptokinase can proteolytically activate plasmin to cleave HA. Host range restriction, replication kinetics, and virulence are also influenced by multiple other genes. Apoptosis appears to be an important mechanism for inducing cell death by influenza viruses and is mediated by several viral proteins, including PB1-F2, NA and NS1 (8).

Influenza viruses have evolved multiple immune evasion mechanisms. For example, the multifunctional NS1 protein counteracts the double-stranded-RNA-activated protein kinase and other antiviral responses induced by interferon, limits the induction of IFN-*β*, interacts with the cellular protein nucleolin, and also interacts via its C terminus with PDZ-binding proteins to modulate viral pathogenicity (175). Viral NA may destroy sialic acid-dependent NK cell-activating receptors on HA expressed on the surface of infected cells, and block their clearance (176). The ability of NA inhibitors to block this effect may provide an indirect antiviral effect through NK elimination of infected cells. HA glycosylation is one mechanism of humoral immune evasion, but lectins in respiratory secretions bind to highly glycosylated HA and thus may limit infection to the upper respiratory tract (174). The unique virulence of A(H5N1) viruses appears to be multifactorial, including high HA cleavability, high replication competence related to its polymerase complex (especially the PB2 protein for certain strains), and ability to subvert host innate immune responses (177, 178).

Bacterial Coinfections

Multiple viral, bacterial, and host factors contribute to the increased risk and severity of bacterial respiratory tract infections following influenza (174, 179, 180). Infection damages the bronchial epithelium and disrupts mucociliary clearance, promotes pharyngeal colonization by bacterial pathogens by increasing adherence to epithelial cells, and depresses the chemotaxis and bactericidal activity of alveolar macrophages and polymorphonuclear leukocytes (PMNs) (Table 5). Viral NA activity can expose cellular receptors that enhance binding by pneumococci and meningococci (174). Decreased bacterial clearance results from decreased PMN chemotaxis, intracellular killing, and other functional changes that have been linked to altered signal transduction steps. Purified viral NP inhibits PMN chemotaxis and superoxide production. The production of nitric oxide, which exerts antiviral effects, is depressed in influenza virus-infected macrophages.

Enhancement of adaptive immune responses designed to establish antiviral immune memory suppress innate antibacterial defenses (179). Type 1 IFN impairs macrophage

and neutrophil responses, in part by inhibiting the IL-17 responses of γδ T cells (181). IFN-γ causes inhibition of alveolar macrophage-mediated bacterial phagocytosis. Increased IL-10 production during recovery suppresses bacterial clearance mechanisms.

The mechanisms involved may vary by bacterial pathogen; e.g., influenza suppression of NADPH oxidase reduces *S. aureus* but not *S. pneumoniae* killing in neutrophils and macrophages (182). Certain strains of *S. aureus* and other bacteria secrete proteases that enhance the infectivity of influenza viruses through HA cleavage and induce severe viral-bacterial pneumonia in animals (183, 184).

Host Genetics

A hereditary predisposition to influenza-related death has been postulated, particularly in association with the 1918 pandemic and with avian A(H5N1) infections, but mechanisms have not been elucidated (185). Genetic polymorphisms in IFITM3 (interferon-inducible transmembrane 3) have been found to be associated with both severe A(H1N1) pdm09 (186) and A(H7N9) infections (137). Other genetic changes, including heterozygosity for the chemokine receptor 5 (CCR5) Δ32 allele (187), polymorphisms in *CD55*, which encodes an important complement regulatory protein (188), the C/C genotype in the surfactant protein B gene (189), and certain HLA alleles affecting T cell functions (190), may also be associated with severe A(H1N1)pdm09 disease. Autosomal recessive interferon regulatory factor 7 (IRF7) deficiency from two mutant alleles, leading to reduced type I and III interferon (IFN) responses, has been reported in an otherwise healthy child with life-threatening A(H1N1)pdm09 disease following primary infection (191). Low mannose-binding lectin production may increase the risk for invasive MRSA coinfection (192). Mutations in the gene encoding carnitine palmitoyltransferase II (*CPT2*) have been associated with influenza-related encephalopathy in Asian children (193).

Immune Responses

Recovery from infection and protection against reinfection are associated with specific host immune responses (194). The observation that the 1977 A(H1N1) reemergence rarely affected persons who had been infected with essentially the same virus more than 20 years earlier indicates that immunity to reinfection and illness caused by homotypic virus is durable. Serologic evidence of reinfection by the same, or closely related, strains is common, especially in closed populations, but reinfections are usually subclinical in adults. The first lifetime influenza A virus infection results in immune memory for the subtype to which the strain belongs, such that subsequent infections or immunizations reinforce the antibody response to the first virus. This phenomenon has been termed "original antigenic sin" (195). Upon subsequent infections, persons may produce cross-reacting antibodies to the subtype causing initial infection, as well as strain-specific antibodies to previously encountered related viruses.

General

Nonspecific host factors may contribute to protection or early response to influenza infection. Various lectins, including mucins in nasal secretions, surfactant proteins, and serum mannose-binding lectin, directly inhibit influenza viruses through binding to their surface glycoproteins, as well as by promoting uptake by phagocytic cells (196). Neutrophil-derived α-defensins inhibit influenza virus replication *in vitro*, possibly by interfering with protein kinase C activation (197). Neutrophils have antiviral effects in animal models, and neutropenic hosts are at increased risk of severe influenza. The pattern of HA glycosylation influences viral opsonization and complement-mediated lysis of infected cells. Human BAL fluids also inhibit HA activity.

Nasal IFN levels peak early and correlate directly with viral titers in uncomplicated influenza. Of note, deficient IFN responses in macaques infected by the 1918 virus (170), lack of detectable lung IFN in fatal pneumonia cases (198), and low plasma IFN-α levels in severe A(H1N1)pdm09 infections (199) indicate the importance of endogenous IFN responses. Type I IFNs cause Mx protein expression that leads to species-specific cellular resistance to influenza virus infection (168) and upregulation of multiple other antiviral pathways (see Chapter 16). Whole-blood gene expression microarrays indicate that influenza patients have attenuation of NK and T cell responses and that both interferon and ubiquitination signaling are strongly decreased in patients with severe outcomes, suggesting the protective roles of these pathways in disease pathogenesis (200). Influenza virus can infect and cause apoptosis and reduced responsiveness of natural killer (NK) cells (511); marked reductions in natural killer (NK) cells have been seen in severe A(H1N1)pdm09 cases (201). In murine models depletion of polymorphonuclear leukocytes is associated with increased influenza replication and worsened disease (202). Apoptosis-dependent phagocytosis by macrophages of virus-infected cells may contribute to elimination of virus.

Humoral Immunity

Following primary infection in children, serum hemagglutination inhibition (HAI) and anti-NA antibodies develop within 10 days and persist for years. The principal neutralizing antibody responses are strain-specific serum immunoglobulin M (IgM) and IgG antibodies and nasal IgA antibodies directed against HA. Virus-specific serum IgM, IgG, and IgA antibodies are present in adults within the first week after the onset of illness (203). Serum HAI titers gradually decrease over the first 6 months but may be boosted by infection with related viruses; IgA responses remain detectable for several months. Otherwise healthy persons infected with avian A (H5N1) virus produce serum-neutralizing antibody responses with kinetics similar to those following primary infection in children with human influenza A viruses.

Protection against infection and illness by the homologous virus correlates directly with levels of neutralizing IgA in nasal secretions and of serum IgG-neutralizing or HAI antibodies (194). Serum HAI antibody titers of 1:32 to 1:40 generally correlate with approximately 50% protection against illness due to the homotypic strain in immunocompetent persons (204), but protective titers vary widely with virus strain, age, and general immune status, and likely intensity of virus exposure. In one household-based study an HAI titer of 1:40 was associated with only 31% protection and a microneutralization (MN) titer of 1:40 with 49% protection against seasonal influenza A virus infection (57).

Antibodies in either serum or nasal secretions confer resistance regardless of subclass if present in sufficient titer. Heterosubtypic humoral immunity, as assessed by HAI titers, provides no significant protection against infection. Non-neutralizing antibodies, which increase phagocytosis of opsonized viruses by Fc receptor-bearing cells, occur following primary infection and may contribute to viral

TABLE 5 Factors Contributing to Bacterial Coinfections with Influenza[a,b]

Disruption of mechanical clearance mechanisms
Epithelial damage leading to loss of mucociliary clearance
Eustachian tube dysfunction
Loss of surfactant
Increased bacterial adherence and replication
Exposure of bacterial receptors (e.g., platelet activating factor receptor, laminin, type I and IV collagen, others)
Viral NA exposure of receptors
Prolonged bacterial colonization and increased replication in the nasopharynx
Favorable milieu for bacterial replication (e.g., serum, fibrinous exudates, erythrocytes, dead cells in airways and alveoli)
Inefficient host clearance of bacteria
Viral depletion of alveolar macrophages
Impaired neutrophil and macrophage bacterial killing (see below)
Impaired NK cell recruitment and function
Downregulation of chemokines and inhibition of immune cell recruitment
Anergy of bacterial sensing pattern recognition receptors (e.g., the Toll-like receptors [TLRs])
Immune dysregulation with increased inflammatory and anti-inflammatory responses
Type 1 IFN impairment of macrophage and neutrophil responses, in part by inhibiting IL-17 responses of γδ T cells
Type II IFN impairment of alveolar macrophage phagocytosis
Bacterial cytotoxins (e.g., pneumococcal pneumolysin. Panton-Valentine leucocidin) increase tissue damage and inflammation
Synergy between influenza and bacteria in activating many of the same cytokines, inflammatory cells, and pattern recognition receptors
Increased interleukin-10 (IL-10) production.
Increased viral replication and reduced viral clearance mechanisms
Bacterial enzymes that cause cleavage activation of viral HA (e.g., *S. aureus*)
Bacterial neuraminidases expose receptors, cleave sialic acids from protective mucins, and possibly overcome inhibitory effects of viral NA inhibitors (e.g., oseltamivir)
Bacterial interference with antiviral immunity

[a] *In vitro* and animal model studies have shown that many factors contribute to enhanced influenza viral-bacterial disease in acute otitis media and pneumonia (174, 179, 180).

[b] Adapted from Hayden FG and de Jong MD. Table 24.2, page 378 in Chapter 24 in *Textbook of Influenza*, Second Edition, Wiley Blackwell; John Wiley & Sons, Ltd., 2013 with permission.

clearance by macrophages. In addition, subtype-specific antibodies, which mediate cell lysis by complement binding or by antibody-dependent cellular cytotoxicity (ADCC), develop after infection or immunization. Antibody to M2 protein is nonneutralizing but inhibits virus replication by ADCC, complement-dependent cytotoxicity, interfering with virus budding, and perhaps other mechanisms (14). Antibody to neuraminidase is not known to neutralize virus infectivity but prevents release of virus from infected cells (205). If present in sufficient titers, anti-NA antibodies can reduce influenza virus replication and prevent illness in experimentally infected animals and humans (206).

Cell-Mediated Immunity

T-cell immunity plays an essential role in influenza immunity, and infection elicits cell-mediated immune responses that are detectable before the appearance of humoral ones (207). Virus-specific lymphocyte blastogenic responses, T-lymphocyte cellular cytotoxicity, and cutaneous delayed-type hypersensitivity are detectable within 6 days after infection. These responses, particularly cytotoxic T lymphocytes (CTLs), play a role in termination of virus replication and recovery from infection, as well as in modifying the risk of illness or complications. Depressed mitogen-stimulated blastogenic responses may occur early in illness and are accompanied by cutaneous anergy. Lymphopenia with decreases in both T- and B-cell counts with normal CD4/CD8 ratios occurs early during acute infection. Decreased mitogenic responses may persist for 4 weeks after infection. T cell anergy has been associated with severe A(H1N1)pdm09 illness during the acute phase of infection (199).

Human CTLs are generally type specific but cross-reactive for cells infected by different influenza A virus subtypes (208). Influenza specific CD8+ T cell responses are directed primarily to the internal proteins (NP, M1, and PB1), although the immunodominant epitopes differ among individuals. CD8+ T-lymphocytes contribute to heterosubtypic immunity in influenza A and, for influenza B, show cross-lineage reactivity (209, 506). CD4+ T cells can also kill virally infected cells directly and recruit other immune cells by producing cytokines; M1 and NP also appear to be immunodominant targets of CD4+ T cells (210). In one experimental study of seronegative volunteers, the presence of influenza A virus-specific memory CD4+, but not CD8+, T cells correlated with lower virus shedding and less illness upon seasonal virus reinfection (211). However, during the 2009 pandemic, higher frequencies of preexisting IFN-γ+ IL-2–T cells to conserved CD8 epitopes were found in individuals who developed less severe illness; CD45RA+ CCR7– late-effector T cells appeared to be the cellular immune correlate of protection (212). Human $CD8^+$ and $CD4^+$ CTLs recognize epitopes of animal influenza viruses (213), and nonexposed healthy adults possess CTLs reactive to A(H5N1) and A(H7N9) viruses that may provide some degree of protection (214, 215). T-cell epitopes are under selective pressure, and substitutions may allow escape from immune recognition (505). Granzyme B elaboration in virus-stimulated peripheral-blood mononuclear cell cultures of CTL activity appears to be lower in immunized elderly adults who develop influenza than those who do not (321). The half-life of influenza CTL memory has been estimated to be 4 years.

CLINICAL MANIFESTATIONS

Influenza causes illness in the majority of those with virus-positive infection (216), but one-half or more of serologically proven infections in adults may be subclinical (217). Infection results in a range of clinical syndromes, including common cold, influenza-like illness (ILI), pharyngitis, tracheobronchitis, pneumonia, and, in children, bronchiolitis or croup. Other respiratory viruses cause ILI (218) and similar syndromes (see Chapter 2). Influenza A and B viruses cause similar illness manifestations and outcomes in ambulatory (219) and hospitalized patients (220). A wide range of acute and sometimes later-onset complications are recognized with such influenza (Table 6) (222), and particular host groups are at higher risk of complications (Table 7) (221).

TABLE 6 Influenza-associated complications[a]

Upper Respiratory Tract
Acute otitis media, sinusitis
Lower Respiratory Tract
Acute bronchitis
Exacerbations of asthma, chronic bronchitis, cystic fibrosis, or other chronic obstructive airways disease
Acute bronchiolitis, obliterative bronchiolitis in children
Laryngotracheobronchitis (croup) in children
Viral pneumonia, acute respiratory distress syndrome (ARDS)
Bacterial pneumonia, empyema, lung abscess, aspergillosis
Bacterial tracheitis
Pneumothorax, pneumomediastinum
Pulmonary fibrosis, bronchiolitis obliterans organizing pneumonia (BOOP)
Central Nervous System
Febrile seizures (children)
Encephalopathy,[b] delirium, acute psychosis, mutism
Acute hemorrhagic leukoencephalopathy
Meningoencephalitis; transverse myelitis; cerebellitis
Hemiballismus; Alice in Wonderland syndrome
Immune-mediated postinfectious encephalitis
Guillian-Barré syndrome; polyneuropathy
Bacterial meningitis, brain abscess
Transient ischemic attack, stroke, vasculitis
Reye's syndrome
Narcolepsy (uncertain)
Parkinson's disease (uncertain)
Cardiovascular
Electrocardiographic abnormalities
Exacerbation of congestive heart failure
Myocardial infarction, acute ischemia
Atrio-ventricular block; arrhythmias; sudden death
Myocarditis, pericarditis
Fulminant heart failure
Cardiac tamponade
Musculoskeletal
Myositis, compartment syndrome
Rhabdomyolysis, myoglobinuria
Rectus abdominis muscle tear
Hematologic
Disseminated intravascular coagulopathy (DIC)
Hemophagocytic syndrome, thrombotic thrombocytopenic purpura
Hypogamnmaglobulinemia, aplastic anemia (uncertain)
Renal
Renal failure from myoglobinuria, DIC, hemolytic uremic syndrome
Exacerbation of nephrotic syndrome
Glomerulonephritis (uncertain)
Gastrointestinal
Reye's syndrome
Parotitis
Gastritis, duodenitis, hematemesis, bowel ulceration
Transaminase elevations
Maternal/fetal
Low birth weight
Fetal distress, spontaneous abortion, stillbirth
Preterm labor, premature delivery
Systemic/Other
Hypotension, sepsis syndrome
Toxic shock syndrome from secondary staphylococcal or streptococcal infection
Poor diabetes control, adrenal hemorrhage
Alopecia areata
Transplant organ rejection
Conjunctivitis, retinitis, choroiditis (uncertain) optic neuritis

[a]Adapted from Hayden FG and de Jong M, Chapter 24 in Webster RG, Monto AS, Braciale TJ, and Lamb RA (Eds), *Textbook of Influenza*, Second Edition, 2013, John Wiley and Sons, Ltd., p 374, with permission. Also see refernce 222.

[b]Described syndromes include acute necrotizing encephalopathy (ANE), acute encephalopathy with biphasic seizures and late reduced diffusion (AESD), clinically mild encephalitis/encephalopathy with a reversible splenial lesion (MERS), posterior reversible encephalopathy syndrome (PRES) (276).

The incubation period of seasonal and pandemic influenza averages about 2 days with a range of 1 to 5 days. However, the average incubation period of avian influenza infection is often several days longer and may extend up to 10 days, or, rarely, longer (115, 121).

Influenza Syndrome

Classic influenza presents abruptly with prominent systemic symptoms, including fever, malaise, headache, and myalgia, and with respiratory symptoms of cough and often sore throat (222). In household-based studies the most common symptoms at influenza onset are cough, runny nose, and sore throat with fever present in about 30% of cases (48). Fever may be quite high and may be continuous or intermittent, especially if antipyretics are used. Pharyngeal and conjunctival injection, minor cervical adenopathy, and clear nasal discharge are common, but physical findings are generally nonspecific. Fever and systemic complaints usually abate by 3 to 5 days in adults, but respiratory complaints increase, with dry cough, substernal burning, and nasal congestion. Slight neutrophilia and lymphopenia occur early, followed by neutropenia. Influenza is associated with elevations of acute-phase proteins, serum amyloid A, and C-reactive protein, especially in hospitalized elderly persons. Acute influenza impairs psychomotor performance and lengthens reaction times.

Recovery is often slow; cough and malaise commonly persist for 2 to 4 weeks. Apparently uncomplicated influenza is often associated with prolonged abnormalities in gas exchange and pulmonary mechanics indicative of small-airway dysfunction (223, 224). These may contribute to the asthenia and decreased exercise tolerance reported by patients during convalescence. The frequency and severity of clinically apparent influenza are greater in smokers (225–227). Allergic patients also experience increased severity of acute symptoms, bronchospastic exacerbations, and prolonged convalescence. Premorbid psychological status correlates with prolonged convalescence. Obesity (body mass indices [BMI] ≥ 30 kg/m^2) and especially morbid obesity (BMI ≥ 40), recognized initially as risk factors for severe influenza during the 2009 pandemic (226, 228, 229), are also associated with increased respiratory hospitalizations

TABLE 7 Groups at increased risk of serious influenza complications[a]

- Persons aged 65 years and older
- Children younger than 2 years of age[b]
- Persons with chronic pulmonary (including asthma and likely chronic cigarette smokers), cardiovascular (except hypertension alone), renal, hepatic, hematological (including sickle cell disease), metabolic disorders (including diabetes mellitus)
- Persons with neurologic and neurodevelopment conditions (including disorders of the brain, spinal cord, peripheral nerve, and muscle such as cerebral palsy, epilepsy [seizure disorders], stroke, intellectual disability [mental retardation], moderate to severe developmental delay, muscular dystrophy, or spinal cord injury)
- Persons with immunosuppression, including that caused by medications or by HIV infection
- Women who are pregnant or postpartum (within 2 weeks after delivery)
- Persons aged <19 years who are receiving long-term aspirin therapy (due to risk of Reye's syndrome)
- American Indians/Alaska Natives, other indigenous groups
- Persons who are morbidly obese (i.e., body-mass index [BMI] ≥40)[c]
- Residents of nursing homes and other chronic care facilities
- Those infected by zoonotic influenza viruses (e.g., avian H5N1 or H7N9)
- Those with genetic predisposition (e.g., IFITM3 polymorphisms)

[a]Adapted from *Antiviral Agents for the Treatment and Chemoprophylaxis of Influenza: Recommendations of the Advisory Committee on Immunization Practices* (ACIP), 2011 (221) with permission.

[b]All children aged <5 years are considered at higher risk for complications from influenza, but the highest risk is for those aged <2 years; the highest hospitalization and death rates occur among infants aged <6 months.

[c]Increased risk of complications has been reported at lower BMI (≥30) in some studies (227–230).

during seasonal influenza (230). Increased influenza-associated pneumonia risk is reported in markedly underweight (BMI <18.5) persons (231). Severity also relates to virus type; A(H3N2) subtype infections have been associated with higher frequencies of lower respiratory symptoms, pulmonary function changes, physician visits, and hospitalizations than seasonal A(H1N1) subtype infections.

Influenza C virus has been associated with 3.5% of common colds in adults (232) and may cause febrile bronchitis and influenza-like illnesses, as well as a range of syndromes, including febrile coryza, bronchiolitis, and pneumonia, in children (102). Rhinorrhea and cough are the most commonly recognized symptoms and may last several weeks.

The diagnosis of influenza is often based on clinical and epidemiological grounds in the context of a known outbreak. In adults presenting with recent-onset fever and cough during community outbreaks, a suspected influenza diagnosis has been confirmed virologically in up to 80% (233, 234). The absence of fever, cough, or nasal congestion decreases the likelihood of influenza. However, clinical diagnosis often lacks accuracy, especially in children below the age of 5 years or when influenza prevalence is low, since the acute respiratory symptoms of influenza mimic those of other viral infections, including those due to respiratory syncytial virus, parainfluenza virus, and adenoviruses. Many influenza virus-positive patients, including those with high-risk conditions or requiring hospitalization, lack typical influenza-like illness and do not receive a clinical diagnosis of influenza (235). Fever may be the only initial manifestation in nosocomial influenza.

Specific Populations

Infants and Children

Influenza virus infection occurs in about one-third of infants during the first year of life and is associated with increased severity because of their lack of immunity and their small-caliber airways. Maternally derived antibodies provide some protection in infants, although influenza hospitalization rates are highest in children younger than 6 months and progressively decrease thereafter (236). Approximately 40% of initial infections in young children are subclinical or cause afebrile upper respiratory tract illness (512). About 90% of symptomatic patients have fever, cough, and rhinitis, up to 40% have emesis or diarrhea, and 25% or more have otitis media or lower respiratory tract disease (98). Pneumonia develops in 5 to 15% of young children. Underlying conditions, like bronchopulmonary dysplasia and congenital heart or neurologic disease, are present in a minority. In adolescents, otitis media, usually resolving without antibiotics, and pneumonia complicate about 5% of cases. More than 1% of infections in the pediatric population result in hospitalization (236). Influenza A and B virus infections have been associated with 68% and 36%, respectively, of croup admissions and 36% and 11%, respectively, of all pediatric hospitalizations for respiratory illness during epidemic periods.

Influenza is underrecognized in infants and young children (98). Unexplained fever or suspected sepsis, bronchiolitis, croup, vomiting, diarrhea, and neurologic manifestations, including apnea, seizures in up to 36%, and meningitis-like presentations, lead to hospitalization. Abdominal pain can mimic acute appendicitis. Myositis, usually manifested as calf tenderness and pain impeding ambulation for hours to several days, occurs in about 20% of influenza B virus-infected children. Myositis and gastrointestinal symptoms are associated more frequently with influenza B than with influenza A virus infections. A range of acute CNS syndromes have been described in influenza-infected children (Table 6) (see below). Abnormal transaminases without the hyperammonemia or hypoglycemia typical of Reye's syndrome may occur. The incidence of Reye's syndrome in children with influenza, estimated to be less than 1 case per 10^5, has markedly decreased in temporal association with the reduced use of salicylates (237).

Pregnancy

Approximately 5 to 10% of pregnant women have serologic evidence of influenza virus infection. Excess mortality during pregnancy, primarily due to overwhelming pulmonary disease, has been well-documented during pandemics and sporadically during epidemics (238). In the 2009 and past pandemics as many as 10% of influenza deaths have been among pregnant women, as well as increases in hospitalization and ICU admissions (239, 240). Increased risks of complications and hospitalization (two- to fourfold) occur with increasing stage of pregnancy in women with seasonal influenza, especially in those with comorbidity (241). The second and especially third trimester of pregnancy or early puerperium appear to be periods of increased risk for severe disease and viral pneumonia. Maternal infection has also been associated with preterm delivery and perinatal mortality, primarily because of an increased rate of stillbirths, fetal distress, and emergency cesarean delivery, which were reported frequently during the 2009 pandemic (240, 242). Progressive cardiopulmonary changes during pregnancy including elevation of the

diaphragm, increased respiratory rate, increased intra-abdominal pressure, and decreased chest compliance may increase the risk of respiratory compromise and possibly pulmonary edema (243). Immunologic changes including increased expression of some proinflammatory cytokines, suppression of IFN-γ, and relative immunosuppression related to fetal tolerance likely contribute to the increased risk of severe disease, particularly during the third trimester (243).

First trimester influenza may be associated with congenital anomalies including neural tube defects that have been related to maternal hyperthermia (244). Influenza during pregnancy has been linked to an increased risk of bipolar disorder in offspring (245). Reported associations with an increased risk of childhood leukemia, schizophrenia (1957 pandemic), or Parkinson's disease remain to be proven (238). Transplacental spread of virus has been documented rarely. A (H5N1) disease is associated with high mortality rates, fetal loss, and transplacental dissemination of virus (121, 155).

Elderly

Viral replication and duration of illness may be more prolonged in older adults, especially the frail elderly, and the risks of acute complications and long-term reduction in functional status are increased (246). Elderly patients with influenza may not complain of typical systemic symptoms. Lassitude, lethargy, confusion, anorexia, decreased activity level, cough, and low-grade fever may be the primary findings (222). Presentation with complications, such as bacterial pneumonia or exacerbations of underlying conditions, also occurs. The elderly may experience myalgia or muscle weakness profound enough to impair ambulation in association with high creatine phosphokinase (CPK) levels (247). In murine models influenza leads to mobility impairments with greater and more prolonged induction of inflammation, atrophy, and proteolysis genes in aged compared to young animals, although without direct evidence of muscle infection (248).

Immunocompromised Hosts

Although not at increased risk of infection, HIV-infected persons and other immunocompromised hosts may have more severe and prolonged illness; viral shedding for months has been observed in those with advanced immunodeficiency (150). An increased frequency of cardiopulmonary complications and hospitalizations occurs in HIV-infected persons, and persons with AIDS experience excess pneumonia and influenza-related mortality during epidemics (249, 250). Influenza in HIV-infected children has been associated with an eightfold-increased risk of hospitalization for lower respiratory illness. In South Africa HIV-positive adults, 5 to 64 years of age, have been estimated to account for 28% of seasonal influenza mortality (251), and increased mortality has occurred in HIV-infected pregnant women during both seasonal influenza and the 2009 pandemic (252).

Influenza virus infections in cancer patients have been associated with variable prolongation of clinical course, increased hospitalizations, and interruption of chemotherapy. Acute-leukemia patients with chemotherapy-induced neutropenia are at high risk of pneumonia and death following influenza. Chronically immunosuppressed solid organ transplant (SOT) and hematopoietic stem cell transplant (HSCT) patients are at increased risk for influenza complications, prolonged virus replication, and emergence of antiviral drug-resistant variants. (253, 254). They may present with few symptoms initially but subsequently progress. Most solid organ transplant patients have self-limited infections, but fever may be prolonged and hospitalization frequent. Infection in transplant patients has been linked with rejection, graft loss, and possibly hemolytic-uremic syndrome. Up to 50% of HSCT recipients develop lower respiratory tract disease, and up to 20% die without antiviral therapy, with the highest risk occurring during the period of aplasia.

Complications

Influenza complications are common and may be manifested in the upper (otitis media and sinusitis) or lower (bronchitis, croup, and pneumonia) respiratory tract, as exacerbations of preexisting chronic diseases (e.g., asthma, chronic obstructive pulmonary disease, cystic fibrosis, diabetes mellitus, and congestive heart failure) or, less often, in extra-pulmonary sites or systemically (222, 255) (Table 6). The most common complications are bronchitis in adults, which occurs in as many as 20% of patients seeking care, and otitis media in children. Influenza is linked to approximately 10% of community-acquired pneumonias in adults, and about 30% of adults hospitalized with seasonal influenza have radiographic evidence of pneumonia (256, 257). A wide range of bacterial co-infections has been reported in hospitalized influenza patients (2–65%) depending on age, co-morbidities, prior antibiotic receipt, and illness severity (513). In patients with reactive-airway or chronic obstructive pulmonary disease, influenza is an important cause of exacerbations, and most illnesses are associated with spirometric deteriorations, usually lasting less than 3 months. Exacerbations of asthma, with FEV_1 (forced expiratory volume in 1 second) decreases lasting 2 to 9 days, occur in most cases of clinically apparent influenza. In patients with cystic fibrosis, influenza virus infections are associated with increased hospitalizations and disease progression, including decreases in spirometry.

Viral Pneumonia

Influenza A, and less often influenza B, virus can cause severe primary viral pneumonia in those with underlying conditions and in previously healthy persons (256). Approximately 15 to 20% of young adults with influenza developed pneumonia in 1918, with associated fatality rates of 30% or higher; bacterial pathogens were detected from the lungs or blood in most fatal cases (169). During the 1957 pandemic, approximately 30% of fatal cases had influenza viral pneumonitis and/or tracheobronchitis without coexistent bacterial infection (258). Rapidly progressive viral pneumonia leading to acute lung injury and often refractory hypoxemia has been well-documented following A(H1N1)pdm09 infection (239). Mild forms of viral pneumonia with patchy radiographic infiltrates are more common, particularly in children, than severe primary influenza viral pneumonia. The latter occurs in 2 to 18% of adults hospitalized with pneumonia during epidemic periods. More than 90% of cases have been linked to influenza A virus infection, and most recognized cases occur in those over the age of 40 years. Various risk factors, including underlying cardiopulmonary disease, rheumatic heart disease (particularly mitral stenosis), malignancy, organ transplantation, corticosteroid or cytotoxic therapy, pregnancy, and possibly HIV infection, have been identified.

Patients present with a preceding influenza syndrome, followed by increasing cough, tachypnea, and dyspnea. The interval from onset of illness to disabling pulmonary symptoms is variable (< 1 to 20 days), but most patients deteriorate within 1 to 4 days. Sputum production occurs in about one-half, and hemoptysis occurs in about one-third. Sputum Gram smear may show abundant PMNs without significant numbers of bacteria. The illness progresses over 1 to 4 days to cause severe respiratory failure, associated acute respiratory

distress syndrome (ARDS), and often multiorgan failure. Chest radiographs are nonspecific but typically show bilateral, diffuse mid-lung and lower-lung infiltrates (Fig. 11). Computed tomography is more sensitive than chest radiography at detecting parenchymal changes, which are often peripheral in location, and may show a wide spectrum of abnormalities including airway thickening and dilatation, peribronchial ground glass opacities, bilateral consolidation, centrilobular nodules, and tree-in-bud opacities (Fig. 12) (259, 260). The course may be protracted, but clinical and radiographic improvement usually occurs within 2 to 3 weeks in survivors. Presentation as Goodpasture's syndrome or a syndrome mimicking pulmonary embolism has been described. Bronchiolitis obliterans with organizing pneumonia, pulmonary fibrosis, and chronic functional impairment may develop in survivors.

Progressive pneumonia with development of ARDS and often multiorgan failure occur in the majority of hospitalized patients with avian A(H5N1) and A(H7N9) disease (Table 3) (115, 121). Similar patterns of leukopenia, lymphopenia, thrombocytopenia, and elevated aminotransferases, creatine phosphokinase (CPK), and LDH are seen in such patients (135). The median times from illness onset to presentation for care and to death are about 4 and 9 days in A(H5N1), illness, respectively.

Secondary Pneumonia

Secondary bacterial pneumonias account for about 25% of influenza-associated deaths in interpandemic periods and were found in about 70% of patients with life-threatening pneumonia during the 1957 and 1968 pandemics (258, 261, 262). Reappearance of fever, increased respiratory symptoms, or cough productive of purulent sputum suggests the possibility of superimposed bacterial infection (262), but presentation with bacterial or mixed viral-bacterial pneumonia without a biphasic illness also occurs. The most common bacterial pathogen complicating influenza is *Streptococcus pneumoniae*, but *S. aureus* accounts for 12 to 25% or more of secondary bacterial infections, and *Haemophilus influenzae* is common (262). Group A beta-hemolytic streptococci, gram-negative bacilli, and *Neisseria meningitidis* infections are also seen. Severe pneumococcal pneumonia including empyema and lung abscess has been associated with influenza in previously healthy children. During the 1957 pandemic, *S. aureus* superinfection was the most common cause of fatal respiratory tract disease related to influenza and was associated with mortality rates of 28 to 48% irrespective of age or prior disease. Severe and often fatal cases of community-acquired, methicillin-resistant *S. aureus* pneumonia associated with influenza are being increasingly seen in both children and adults. Fungal infections, particularly *Aspergillus* infections, have been rarely reported in association with cutaneous anergy, lymphocytopenia, and sometimes systemic corticosteroid use. No clear association between preceding influenza and the occurrence of *Mycoplasma pneumoniae* or *Legionella* infections has been found (509). Specific transcriptomic signatures may prove useful in distinguishing influenza from bacterial pneumonia (263, 264). Blood procalcitonin measurement appears helpful in detecting mixed influenza-bacterial infections, in that low levels suggest that bacterial coinfection is unlikely (265).

Extrapulmonary Complications

Other recognized but rare (<1% of cases) complications include a range of CNS syndromes (encephalitis or encephalopathy, meningitis, transverse myelitis, and polyneuritis), acute parotitis, myocarditis and pericarditis, acute myositis, rhabdomyolysis with myoglobinuria, and acute renal failure, disseminated intravascular coagulopathy, arthritis, and Stevens-Johnson syndrome (Table 6) (222). CPK elevations may be as high as 10,000 IU/ml in influenza-associated rhabdomyolysis, which very rarely causes compartment syndromes. Transient subclinical electrocardiogram changes without associated cardiac enzyme elevations or echocardiographic abnormalities, lasting usually 2 weeks or less, occur in as many as one-quarter of adults with apparently uncomplicated influenza (266). While direct myocardial injury is rare in ambulatory adults (267), influenza is associated with increased risk of acute coronary and cerebrovascular events (268–270, 503). Severe cardiac involvement, rarely associated with recovery of virus from the myocardium or blood, has manifested as acute heart failure, pericardial tamponade or effusion, and fatal arrhythmia (271). Hepatic decompensation may occur in those with preexisting liver disease.

Acute CNS manifestations include seizures, coma without focal signs, delirium, behavioral disturbance, cerebellar signs, and increased intracranial pressure (272, 273). These events are more common in children but also occur in adults (274); both influenza A and B viruses have been implicated (275). Acute influenza encephalopathy in children is typically manifested by fever, seizures, altered mental status, and often rapidly progressive coma, usually within 5 days of illness onset. Multiple syndromes have been described (Table 6) (276), and less common findings include thalamic necrosis, increased CSF protein or pleocytosis, and presence of viral RNA in the CSF. Full recovery is usual in milder cases but necrotizing encephalopathy is associated with mortality >25% (276). Virus has rarely been isolated antemorten from the CSF or brain, although CNS dissemination has been documented in A(H5N1) disease (145).

Postinfluenzal encephalitis begins 1 to 3 weeks after the illness and is ascribed to an autoimmune process with demyelination and vasculopathy. Patients develop fever and decreased consciousness or coma in association with lymphocytic pleocytosis and diffuse slowing on electroencephalograms. Encephalopathic symptoms usually resolve in 2 to 25 days. Influenza may uncommonly trigger Guillain-Barré syndrome (GBS) (277) and possibly narcolepsy (278). Possible linkage of influenza to delayed-onset encephalitis lethargica and postencephalitic parkinsonism remains to be proven (279–281).

Toxic shock syndrome may follow within 1 week of onset of influenza and has been linked to either respiratory tract colonization or infections, including sinusitis, pneumonia, or enterocolitis, with toxigenic *S. aureus* strains or group A streptococci (507). Influenza outbreaks are associated with an increased risk of invasive meningococcal disease (282), possibly related to virus-induced mucosal damage or immunosuppression. Cases usually occur within 2 weeks following influenza. Both influenza A and B virus infections have been associated with theophylline toxicity related to decreased clearance.

Thogoto and Dhorl Viruses

The small number of recognized human infections have been associated with meningitis, encephalitis, or systemic febrile illness, including rash, thrombocytopenia, leukopenia, and multiorgan failure (9).

LABORATORY DIAGNOSIS

Influenza diagnostic testing is possible with a wide variety of laboratory assays (42, 283) (see Chapter 15). Testing is

generally indicated if the findings would result in a change in clinical management or have public health implications. Consequently, testing is clearly warranted in seriously ill or hospitalized patients, investigation of unexplained illness clusters and nosocomial outbreaks, and in patients with possible zoonotic infection by novel strains (e.g., history of exposure to swine, poultry, or markets or travel to areas with enzootic influenza). Under such circumstances molecular detection methods are preferred because of their greater sensitivity. However, single negative RT-PCR results do not exclude influenza, and, for critically ill patients with suspected influenza, testing additional respiratory specimens from multiple sites, especially lower respiratory tract ones, is important.

Specimen Collection and Transport

Detection of influenza in clinical specimens depends on sample type and quality, duration of illness, patient age, and influenza virus strain. Influenza viruses can be readily isolated early in illness from various respiratory specimens, including nasopharyngeal swabs, nasal aspirates or washes, sputum, and tracheal aspirates. Throat swabs or washings contain lower virus concentrations and are usually less sensitive than nasal samples, except in sporadic A(H5N1) disease, in which the converse holds (147). Nasopharyngeal swabs or combined nose and throat swabs are reasonable specimens for upper respiratory tract sampling in uncomplicated illness, but lower respiratory ones (i.e., tracheal aspirates, bronchial washings) are advisable for seriously ill patients with lower respiratory disease. One recent study reported that upper respiratory tract samples were negative in 43% of critically ill influenza patients positive for virus in the lower respiratory tract (284). Upper respiratory specimens should be collected as soon as possible, preferably less than 3 to 4 days after illness onset. A swab with a wood shaft should not be used for respiratory specimen collection because it may interfere with molecular assays.

Samples for molecular testing can tolerate a broader range of transport conditions. Because freezing, especially in standard −20°C freezers, of specimens may cause greater loss of infectivity than short-term storage at 4°C, refrigerated samples should be transported for processing within 1 to 4 days for virus isolation. Freezing at or below −70°C more effectively preserves infectivity.

Virus Isolation

Embryonated hen's eggs are a practical isolation system but may be less sensitive than cell culture for many contemporary human influenza viruses (42, 285). Primary rhesus or cynomolgus monkey kidney cell cultures are sensitive for most strains. Several continuous epithelial cell lines, particularly MDCK and the rhesus monkey kidney-derived LLC-MK2, are useful for primary isolation in conjunction with incorporation of trypsin into serum-free medium to effect proteolytic activation of HA. Because of its relative temperature stability, 1-tosylamide-3-phenylethylchloromethyl ketone-treated trypsin (TPCK trypsin) is recommended. MDCK cells are comparable in sensitivity to primary rhesus monkey kidney cells for most influenza virus strains. MDCK cells that are stably transfected to overexpress *a*2,6-linked sialic acid receptors appear to be useful for both *in vitro* susceptibility testing and isolation of virus from clinical specimens (286). Other cell types (e.g., Vero, mink lung, and MRC-5 human embryonic lung cells) will support primary isolation if trypsin is used. Isolation may be facilitated by incubation at 33°C, rolling cell culture tubes, and centrifugation of the sample onto cell monolayers. Laboratory cross-contamination sometimes causes false-positive isolation results.

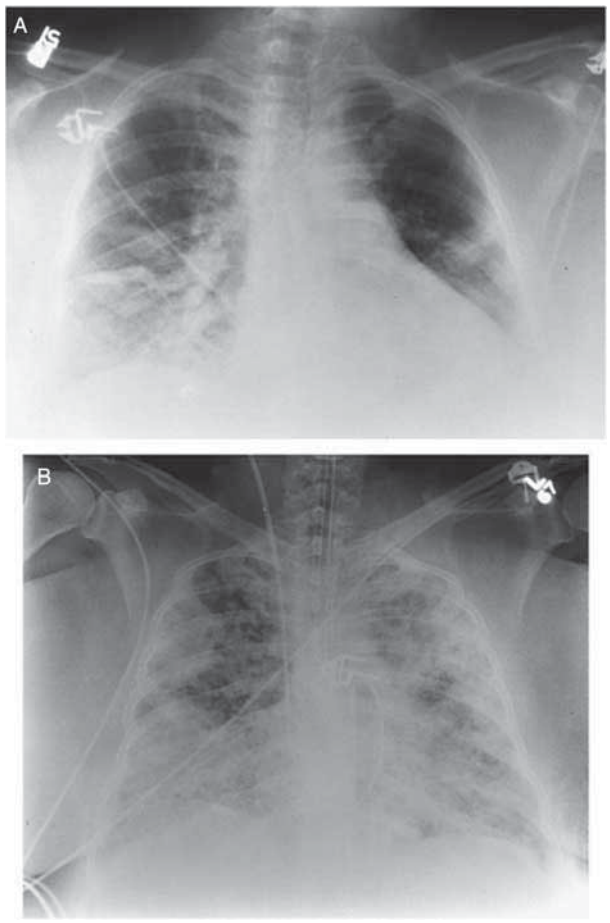

FIGURE 11 Sequential chest radiographs from a 30-year-old nonimmunocompromised female with acute influenza A virus pneumonia. (A) Her symptoms began 1 day before first radiograph, which shows right middle and bilateral lower lobe infiltrates. Her respiratory status deteriorated rapidly, and she required mechanical ventilation but survived. (B) The second radiograph, taken approximately 24 hours after admission, shows diffuse infiltrates.

The CPE, particularly of influenza A viruses, is nonspecific and may be absent or difficult to detect. More than 50% of cultures show CPE within 3 days of inoculation, and more than 90% show CPE within 5 days. Virus replication is usually detected in cell culture by hemadsorption with guinea pig, turkey, or chicken erythrocytes, with detection performed at fixed times after inoculation or when CPE is noted. Because of changes in receptor specificity since 1993, use of chicken erythrocytes is not advised for detection of human strains. Blind hemadsorption of monolayers is positive in more than 85% of samples at 2 days, and nearly 100% of samples at 3 days, after inoculation. For detection of avian influenza viruses or HAI antibody testing, avian or horse erythrocytes are used, since these predominantly express *a*2,3-linked sialic acid receptors.

Identification of isolates can be done by HAI testing using antisera to current strains and appropriate types of erythrocytes or by immunofluorescence (IF) or enzyme immunoassays (EIAs) using type- or subtype-specific antisera. Nucleic acid amplification tests with selected primers and probes or sequence analysis of HA and NA genes provide

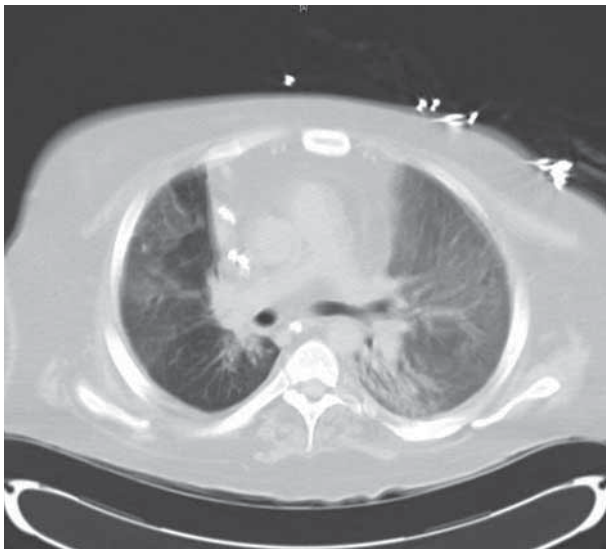

FIGURE 12 Computed tomography of chest without contrast in a HSCT patient who developed severe A(H1N1)pdm09 pneumonia approximately 5 months posttransplantation, while taking prednisone 20 mg/day for chronic GVHD. The study was made approximately 5 days after illness onset and shows diffuse bilateral interstitial opacities, bronchial wall thickening, and a denser left posterior chest consolidation with air bronchograms. Figure courtesy of Dr. Michael Ison, Northwestern University, reprinted with permission.

rapid typing. Rapid detection of influenza virus antigen can also be accomplished by EIA or IF testing of monolayers.

Centrifugation of samples onto MDCK monolayers in shell vials or plates combined with antigen detection has a sensitivity of about 80% (range, 56 to 100%) at 1 or 2 days. Shell vial monolayers of mink lung or rhesus monkey kidney cells may provide greater sensitivity. A commercial mixture of mink lung and A549 cells (R-Mix) in shell vials is useful for detection of influenza and other respiratory viruses.

Primary isolation of influenza C virus has been accomplished in embryonated hen's eggs and a human malignant melanoma cell line (HMV-II) or sometimes MDCK cells, in which hemadsorption is positive with chicken, but not guinea pig, erythrocytes (102).

Antigen Detection

Direct detection of influenza viral antigens in respiratory specimens has been accomplished with IF, EIA, radioimmunoassay, and time-resolved fluoroimmunoassay within 1 to 4 hours. Monoclonal antibodies directed against type-specific NP or M proteins overcome the variable antigenicity of the surface glycoproteins. Direct and indirect IF microscopy of respiratory epithelial cells with commercially available monoclonal antibodies is rapid and has a sensitivity greater than point-of-care (POC) antigen tests (below) (42). Nasopharyngeal aspirates usually have higher cellular content and are superior to swabs; cytocentrifugation may enhance sensitivity.

Multiple commercial EIAs for rapid ($\leq$30 minutes) laboratory-based or CLIA-waived POC diagnosis are available (287). The results are qualitative, and sensitivity depends on sample type and quality, duration of illness, patient age, and influenza virus type. Assay sensitivities are approximately 50 to 70% (range, 10 to 80%) and are higher in children than in adults and higher in influenza A than in B virus infections (285, 288). Nasopharyngeal aspirates and swabs have somewhat higher yields than washing and throat swabs or gargles (285). Approved respiratory specimens vary among the FDA-cleared influenza assays. Clinical sensitivity is highest on days 2 and 3 of illness but decreases rapidly thereafter (288). Analytic sensitivity varies across assays and viruses, in part, related to the ability of the various proprietary monoclonal antibodies used to detect NP differences, and may be lower for A(H1N1)pdm09, swine variant, and some avian influenza viruses (289–291). Mucoid samples, like sputum or tracheal aspirates, cause false-positive or -negative reactions in some assays. Consequently, false negative results are common, especially in adults when influenza activity is high, and negative results should not be used to guide management decisions. Although assay specificities (range, 85–100%) are generally high, false positive results occur, especially during periods of low influenza activity, so that confirmation by RT-PCR or viral culture may be required. The use of objective readout devices (Sofia Influenza A+B and BD Veritor System) help reduce false positive results. Positive results may occur up to one week after intranasal live-attenuated influenza vaccine (292).

Nucleic Acid Detection

Many commercial (293) and in-house RT-PCRs (singleplex and multiplex; real-time and other RNA-based) and other molecular assays are available for influenza virus RNA detection. Depending on the specific assay, a broad range of specimen types can be tested with performance times ranging from 1 to 8 hours. Such assays are more sensitive than virus isolation in detecting influenza virus in clinical samples, in part, because they can detect noninfectious virus RNA, although their higher sensitivity may also overestimate the infectious period (57). Multiplex commercial assays (e.g., FilmArray Respiratory Panel, BioFire Diagnostics, LLC; Verigene Respiratory Pathogen Nucleic Acid Test, Nanosphere, Inc.; x-TAG Respiratory Viral Panel, Luminex Molecular Diagnostics Inc.) can also detect other respiratory pathogens. Multiplex primer combinations can distinguish between A and B types and, in some assays, circulating influenza A H1 and H3 subtypes; positive but nontypable results should prompt consideration of infection by novel strains.

Recently FDA-approved, influenza-specific rapid molecular assays can provide results in less than 30 minutes for POC testing. One (Alere i Influenza A&B) is CLIA-waived for use with nasal swab specimens (294), and another (Roche Cobas Influenza A/B) is CLIA-waived for use with nasopharyngeal swabs. Their reported sensitivities range from 70 to 100% (293).

Lower respiratory tract samples (tracheal aspirates, BAL) have higher yields than upper respiratory tract samples in those with viral pneumonia, especially when due to zoonotic influenza viruses. Only the USCDC RT-PCR assay, available at qualified public health laboratories, is currently FDA-approved for lower respiratory tract specimens. Off-label use of commercial assays is an alternative, but some may lack sensitivity for detecting swine or avian viruses (290). Sensitive multiplex real-time RT-PCR assays provide rapid quantitative detection of influenza A and B virus RNA and can be used in combination with shell vials, which can also provide isolates for analysis (295). Nucleic acid amplification assays with selected primers, or combined with restriction enzyme analysis, can detect influenza A viruses harboring M2 gene mutations associated with resistance to adamantanes or

selected NA mutations associated with resistance to neuraminidase inhibitors. In addition, this approach can differentiate between the genes of vaccine-like strains and circulating ones and provide data about HA genetic drift.

Serology

Serologic studies are not useful for the rapid diagnosis of influenza because most cases are reinfections, and paired acute- and convalescent-phase sera are usually needed. Commonly used assays include complement fixation (CF), HAI, and enzyme-linked immunosorbent assay (ELISA). Less commonly employed tests include neutralization (Nt) or microneutralization, single radial hemolysis, radial immunodiffusion, passive hemagglutination, and NA inhibition. The CF test utilizes type-specific internal antigens (NP), and, unlike the HAI test, it is not influenced by the antigenic variability of the circulating strain or by the presence of serum inhibitors. However, CF is less sensitive than HAI or ELISA and detects a rise in antibody titer in no more than 70% of infections. HAI titers reflect subtype- and strain-specific antibodies directed to HA. HAI testing requires inactivation of nonspecific inhibitors in the sample, and its sensitivity depends on the antigenic variant employed. Sensitivity is enhanced by antigen preparation in cell culture and, particularly for influenza B virus, ether treatment of antigen. Because anamnestic responses frequently occur, inclusion of antigens that resemble circulating strains and those of past prevalent strains increases diagnostic yield. Fourfold-or-greater rises in antibody titer occur in 80% or more of infections. The Nt test is the most specific of the conventional assays, correlates best with protective immunity, and is the current serologic method of choice in A(H5N1) infections but is labor-intensive. Detection of HAI antibodies to H5 with horse erythrocytes or by Nt testing with pseudotyped virus expressing H5 HA may overcome this problem (296). Antibody detection by ELISA is more sensitive than other assays and can be used to measure HA-specific responses or particular antibody types.

PREVENTION

Nonpharmaceutical Interventions

The effectiveness of various nonpharmaceutical interventions like social distancing, hand hygiene, cough etiquette, and masking in influenza prevention has received considerable attention in the context of both seasonal influenza and pandemic response. Timely implementation of multiple public health measures, including combinations of school closures, cancellation of mass gatherings, case isolation, and voluntary quarantine of contacts that were taken in some cities during the 1918 pandemic, appears to have reduced its community impact, although the value of any individual intervention is uncertain (297). Holiday periods are associated with reduced seasonal influenza rates, and prolonged school closures are predicted to reduce peak attack rates and cumulative numbers of cases in children and adults (298). Such interventions are being contemplated as part of a community mitigation strategy in the face of a pandemic or outbreak associated with high mortality.

In community and household settings, reduced risk of virus exposure can be accomplished with adherence to frequent hand hygiene and cough etiquette for those with illness. Soap and water or alcohol-based hand rubs are highly effective in reducing influenza virus on hands (299). Other common sense interventions include minimizing visitors other than necessary caregivers, maintaining adequate ventilation in shared areas, and avoiding exposure of at-risk persons to ill ones (e.g., pregnant women should not provide care for ill persons). The value of hand hygiene alone in preventing influenza transmission remains uncertain and may vary by context. Regular hand hygiene appeared partially effective in Egyptian schoolchildren (300). Early implementations and compliance with both masks and hand hygiene appear to lower the risk of transmission to household contacts (301, 302).

Zoonotic Infections

Avoidance of exposure can reduce the risk for zoonotic infections. Live bird markets are central to human infections by avian influenza viruses, and their closures have been temporally associated with cessation of avian A(H7N9) infections in humans (131, 132). Travelers to countries with known outbreaks of avian influenza should avoid poultry farms, contact with animals in live bird markets, entering areas where poultry may be slaughtered, and contact with any surfaces that may be contaminated with excreta from poultry or other animals. Similarly during swine influenza outbreaks, persons at increased risk for complications from influenza should avoid swine and swine barns at agricultural fairs or other affected sites. Frequent hand hygiene and adherence to good food safety and hygiene practices are always sensible and may contribute to risk reduction.

Nosocomial Infections

Patients hospitalized with human influenza should be managed with standard and droplet precautions, preferably in an individual room. Cohorting of influenza patients may be necessary during high demand periods. When feasible, masking of suspected or proven influenza patients in healthcare settings (e.g., emergency department, during transport, in radiologic facilities) probably reduces environmental contamination and the risk of nosocomial transmission. Surgical masks are effective in preventing the dispersion of influenza RNA-containing droplets from coughing patients (303). For protection of healthcare workers caring for influenza patients, the incremental value of using fit-tested respirators compared with surgical or procedure masks remains uncertain (304). Cloth masks are much less effective than medical masks (305). Under experimental conditions fit-tested N-95 respirator and eye protection are required for protection against exposure to influenza virus in small particle aerosols (306). Respirators should be used in circumstances of aerosol-generating procedures, and eye protection (goggles or face shields) is also warranted. Visitors should be minimized in general during community outbreaks and prohibited if ill. Healthcare worker immunization and, under some circumstances, antiviral chemoprophylaxis are important tools to mitigate the risk of nosocomial transmission (see below).

Inactivated Vaccines

Inactivated influenza vaccines were initially introduced in the 1940s but were impure, reactogenic, and variably potent. During the 1970s, split and subvirion preparations, using embryonated hen's eggs as the substrate, were introduced. In most countries, whole virus vaccines have been replaced by these less reactogenic split virus ones, in which virus has been disrupted by detergent, and subunit ones, in which the HA and NA antigens have been purified by removal of other viral components. More recently, vaccine production in cell

culture, recombinant HA vaccines, and adjuvants including proprietary oil-in-water ones (e.g., MF-59, AS03) have been introduced into clinical use. A wide variety of seasonal vaccines are now approved for use in the United States (Table 8) and many other countries. Additional vaccines, including virosomes and higher-dose intradermal ones, are approved in different countries (307). Antigens for the majority of inactivated vaccines are still mass produced in embryonated chicken eggs by use of high-yield reassortant viruses that express the HA and NA of circulating strains, although cell culture-based production methods are increasing. Residual egg proteins can rarely cause immediate hypersensitivity reactions in those with severe egg allergy and possibly contribute to other adverse effects (see below), but a recombinant HA vaccine devoid of egg proteins is now available (Table 8).

The HA content of inactivated vaccines is standardized at a minimum of 15 μg per antigen for those aged ≥3 years (9 μg for intradermal vaccine), although higher HA dose vaccines are approved in certain age groups (Table 8). Hemagglutination-inhibition (HAI) antibody titers correlate closely with those measured by microneutralization and are the traditional ones used to assess vaccine-induced serologic responses and cross-reactivity to new variants. The composition of influenza vaccines is determined semiannually on the basis of the antigenicity of circulating influenza viruses through the WHO's Global Influenza Surveillance and Response Network (308). Recent seasonal vaccines contain antigens of two influenza A virus subtypes (H3N2 and H1N1) and of one or two influenza B virus lineages. Quadrivalent vaccines provide greater influenza B coverage and are increasingly replacing trivalent ones. The waning of vaccine-induced immunity over time necessitates annual reimmunization even if the vaccine antigens are unchanged. Annual guidelines for immunization are published by the U.S. CDC (Table 1).

Immunogenicity

Inactivated vaccine is highly immunogenic in young adults but less so in the elderly, infants, and persons with chronic illness or immunosuppression, including those with HIV disease, solid-organ and bone marrow transplant recipients, and in those receiving cancer chemotherapy. Immunogenicity is also lower in those with high preexisting antibody levels. Vaccine seroresponses, and perhaps vaccine effectiveness, may be diminished with repeated annual immunization, especially when there are small or no changes in vaccine strains (309–312). Protection against illness correlates with serum HAI antibody levels (generally titers ≥1:40 provide 50% protection against illness in healthy adults), but illness may occur despite high postimmunization HAI titers (313). Parenteral immunization may stimulate limited mucosal antibody production and CTL responses. In primed healthy adults, immunization results in presumably protective levels of serum HAI antibody in >85% for the homologous strain. Because 60% or fewer of unprimed children respond, two doses of vaccine at least 1 month apart are required. Protective HAI antibody responses usually occur within 10 days in responding adults, including those with cardiopulmonary disease. The duration of protection following immunization against homotypic virus is uncertain but may last up to 2 to 3 years. Vaccine-induced antibodies to NA also appear to confer protection against illness (314), but anti-NA responses are seen in less than one-half of vaccine recipients, and the NA content of FDA-approved vaccines is not routinely measured.

Age-related declines in serologic and CTL memory responses to vaccine occur with advancing age, and failure of vaccine boosting occurs more commonly in the elderly. The degree of infirmity, rather than increasing age by itself, is a critical determinant of decreased vaccine responsiveness. Chronic statin use may be associated with reduced antibody responses in the elderly (315). However, vaccine administration in the morning appears to increase immunogenicity (508). A second dose of vaccine at 1 or 3 months does not boost serum HAI titer responses in healthy, elderly persons (316). High dose vaccine containing fourfold-higher antigen content (60 μg of HA) improves immunogenicity in the elderly, HIV-infected persons, and adult oncology patients receiving chemotherapy compared to standard-dose inactivated vaccine (317–319). An MF-59 adjuvanted vaccine, available in multiple countries, appears to induce somewhat higher and broader antibody responses in the elderly than nonadjuvanted vaccine (320) and was approved for this age group in 2015 in the United States (Table 8). T-cell responses may be better correlates of vaccine protection in the elderly than antibody levels (321). While later booster doses do not appear to increase protection in the frail elderly, a second dose may improve immunogenicity in certain high-risk groups (e.g., organ transplant or chemotherapy recipients).

Effectiveness

Influenza virus vaccines vary in their effectiveness depending on the formulation used in a particular year, on the age and health status of the vaccinee, and on the overall virulence of the circulating strains. Seasonal vaccine efficacy in preventing clinical influenza is generally moderate but may be low or absent in some seasons, in part, related to the degree of antigenic match between the epidemic virus and vaccine strains. The efficacy of inactivated vaccines in preventing illness is generally 50 to 70% in adults aged 18 to 64 years, but high-quality data are lacking in the elderly (≥65 years) (322). MDCK-produced vaccine provides similar protection to vaccine from eggs (323). Immunization of ambulatory adults reduces absenteeism and physician visits due to respiratory illness by about 30 to 60% during epidemic periods, as well as the risk of influenza-associated pneumonias (324). Vaccine coverage level among adults aged 18 to 64 years is also associated with reduced influenza risk in the elderly (325).

Inactivated vaccine efficacy in children aged 6 to 72 months is generally low (43% in one major trial) but can be increased by using MF-59 adjuvant (326). Influenza vaccine in children is more efficacious in preventing moderate-severe than mild illness (327). Immunization of children (inactivated or LAIV) reduces the risk of influenza-related otitis media and influenza-associated pneumonia (324), as well as the likelihood of illness in nonimmunized household and community contacts (328, 329). Wide-scale immunization of school-age children may lessen the community impact of epidemic influenza and, in Japan, was temporally associated with reduced respiratory and overall mortality in older adults (330).

Among ambulatory older adults in the community, immunization provides about 50 to 60% protection against influenza, is cost saving, and reduces hospitalizations and mortality during outbreaks (331, 332). During the seasons 2005/06 through 2013/14, immunization was estimated to have prevented a total of 25,694 to 59,210 deaths in the United States (333). Immunization is associated with a lower risk of major adverse cardiovascular events, particularly in those with more active coronary disease (334). Compared to standard vaccine, high-dose vaccine (Table 8)

TABLE 8 Seasonal influenza vaccines approved for use in the United States, 2015–2016 season[a]

Vaccine type designation	Production substrate	No. companies (trade names)	Approved age range (years)	Route of delivery	HA dose (ug) per antigen	Comment
Inactivated influenza vaccine, quadrivalent (IIV4)	Eggs	3 (Fluarix Quadirvalent, FluLaval Quadrivalent, Fluzone, Quadrivalent,	≥0.5[b]	IM (0.25 for 6–35 months, 0.5 ml for >36 months)	15 (for 0.5 ml dose)	
Inactivated influenza vaccine, trivalent (IIV3)	Eggs	3 (Afluria[c], Fluvirin, Fluzone)	≥0.5[b]	IM (per above)	15 (for 0.5 ml dose)	Needle-free jet injector approved 2014 for delivery of Afluria in those aged 18 to 64 years. Potential risk of febrile reactions in children <9 years old with Afluria[c]
IIV3, High-dose	Eggs	1 (Fluzone High Dose)	≥65	IM	60	
IIV3- Intradermal	Eggs	1 (Fluzone Intradermal Quadrivalent)	18–64	ID (0.1 ml by microinjector)	9	
IIV3- Cell culture	MDCK cells	1 (Flucelvax)	≥18	IM	15	Minute quantities (<$5x10^{-8}$ μg) of total egg protein including ovalbumin per dose
IIV3- MF59-Adjuvanted	Eggs	1 (Fluad)	≥65	IM	15	Approved 2015 in the USA
Recombinant influenza vaccine, trivalent (RIV3)	Insect cells	1 (FluBlok)	≥18	IM	45	No ovalbumin or egg protein Expanded age range approved 2014.
Live-attenuated influenza vaccine, quadrivalent (LAIV4)[f]	Eggs	1 (FluMist)	2–49[d]	IN spray (0.2 ml divided between nostrils)	∽10^7 infectious doses per virus	Not approved for pregnant women or those with comorbidities. See contraindications and precautions[d]

[a]Adapted from US Centers for Disease Control and Prevention (341). Contraindications to influenza vaccines include severe allergic reaction to any vaccine component, including egg protein, or after previous dose of any influenza vaccine. Precautions include moderate to severe acute illness with or without fever; history of Guillain-Barré syndrome within 6 weeks of receipt of influenza vaccine.

[b]Approved age range varies with particular product.

[c]ACIP recommends Afluria not be used in children aged 6 months through 8 years because of increased risk of febrile reactions noted in this age group with bioCSL's 2010 Southern Hemisphere IIV3.

[d]ACIP recommends LAIV4 not be used for pregnant women, immunosuppressed persons, persons with egg allergy, and children aged 2 through 4 years who have asthma or who have had a wheezing episode noted in the medical record within the past 12 months, or for whom parents report that a healthcare provider stated that they had wheezing or asthma within the last 12 months.

Persons who care for severely immunosuppressed persons who require a protective environment should not receive LAIV4 or should avoid contact with such persons after vaccination.

[f]Because of LAIV's poor and/or lower than expected vaccine effectiveness in three recent seasons, the ACIP has recommended against use of LAIV in children for the 2016-17 season (CDC Media Statement, 22 June 2016).

is associated with further reductions in influenza illness and associated hospitalizations or emergency department visits in those aged ≥65 years (317, 335). MF-59 adjuvant also appears to increase the protection of inactivated standard vaccine in the elderly (320, 336). Although not approved for use, an AS03-adjuvanted seasonal vaccine also has higher efficacy for prevention of influenza A(H3N2) illness than does a nonadjuvanted one (337).

In nursing home residents, protective efficacy against influenza illness ranges widely and averages only 20 to 45%. However, immunization reduces influenza-related hospitalizations and mortality in such persons. High nursing home immunization rates (≥80% of residents) may indirectly confer protection against outbreaks by increasing levels of herd immunity (106).

Reactogenicity

Injection may cause local redness, tenderness, and induration for 1 or 2 days in as many as one-third of vaccinees. Fever and constitutional symptoms beginning 6 to 12 hours after vaccination and lasting for several days occur in 1 to 5% of adult recipients and more often in young children. Elderly subjects tend to have lower rates of local and systemic reactions, although high-dose and MF-59 adjuvanted inactivated vaccines are associated with higher frequencies of injection site reactions than standard dose vaccine. Because of the potential for causing febrile reactions, only split-virus or subvirion vaccine should be used for seasonal immunization in children. An oculorespiratory syndrome (red eyes, facial edema, sore throat, and respiratory complaints) starting within 2 to 24 hours of immunization and resolving spontaneously within 2 days has been described uncommonly with some vaccine lots but is not a contraindication to immunization (338).

Immediate hypersensitivity reactions (e.g., hives, wheezing, anaphylaxis) are usually secondary to egg protein hypersensitivity, so the vaccine should be used with caution in patients who have documented allergies to eggs or egg products. However, allergic reactions are rare even in those with history of egg allergy (339) and have occurred after receipt of egg-free recombinant vaccine, indicating that that some reactions are not due to egg proteins (340). Those who had serious reactions to eggs (e.g., angioedema, respiratory distress, lightheadedness, recurrent emesis, resuscitation) may receive RIV3 if they are aged ≥18 years (Table 8) and there are no other contraindications (341). Severe allergic reaction to influenza vaccine is a contraindication to future receipt of the vaccine.

A temporal association between immunization and GBS development within 6 weeks was found during the 1977 swine influenza immunization program, during which approximately 430 GBS cases occurred among 41 million vaccinees, a rate estimated to be sevenfold higher than expected (342). Whether influenza vaccines in certain seasons may be associated with a low risk of GBS ($\backsim$1 per 10^6) in older vaccinees is unresolved. The causes of an association between receipt of AS03-adjuvanted A(H1N1)pdm09 vaccine and narcolepsy in genetically predisposed children and adolescents remain under investigation (343, 344). Reported associations of influenza immunization with exacerbations of asthma, systemic vasculitis, recurrent GBS, adverse ocular effects, or pericarditis remain unproven; asthma exacerbations are not associated with inactivated vaccine. No important changes in drug metabolism have occurred with current vaccines. Pneumococcal and influenza vaccines can be given at the same time at different sites. When administered together with either pneumococcal or a diphtheria-tetanus-acellular-pertussis-containing vaccine, inactivated influenza vaccine is associated with a small increased risk of febrile seizures in children aged 6–23 months (510).

Target Populations

The United States adopted a policy of universal influenza immunization for all persons aged 6 months or older and without contraindications in 2010. In other countries, populations targeted for immunization vary but generally include both those at increased risk for influenza-related complications (Table 7) and persons who are in close contact with high-risk individuals, particularly healthcare workers. Healthcare worker immunization probably reduces both absenteeism and the risk of nosocomial transmission with its associated mortality (345, 346). Strategies that increase vaccine uptake in healthcare providers include ready access to no-cost vaccine in the workplace and especially mandatory employer requirement (514).

WHO recommends that pregnant women should have the highest priority for seasonal influenza immunization and that additional risk groups for consideration (in no particular order of priority) are children aged 6 to 59 months, the elderly, individuals with specific chronic medical conditions, and healthcare workers (347). Pregnant women are an important risk group, in whom immunization reduces influenza risk in both mother and newborn (348, 349). Immunization also appears to reduce the risk of stillbirths by about one-half (350). Inactivated vaccines are safe, and pregnant women can be vaccinated at any stage of pregnancy (347). Administration of inactivated vaccine is considered safe during any stage of pregnancy. Foreign travelers, particularly elderly or high-risk persons, should be immunized before travel to the tropics during any time of the year or during April to September if travel is to the southern hemisphere. Immunization is appropriate for anyone who wishes to reduce his or her risk of acquiring influenza.

In HIV-infected persons, advanced disease is associated with low antibody responses, which are not augmented by a booster dose or doubled HA antigen content. Those with CD4 counts of $<100/mm^3$ rarely show antibody rises, but immunization appears to be protective in those with CD4 counts of $>200/mm^3$ despite diminished HAI responses (351). Influenza immunization may be followed 1 to 4 weeks later by transient increases in plasma virus titers in some recipients, but the clinical significance of such changes is doubtful, and no long-term effects on CD4 counts or clinical progression have been recognized (351, 352).

Those with hematologic malignancies, including myeloma, often have reduced responsiveness to vaccine (351). Oncology patients manifest HAI antibody responses less often when they are immunized concurrently with, or shortly after, chemotherapy compared to immunization between courses of chemotherapy. If immunization is required during chemotherapy, a two-dose schedule of vaccination separated by 3 to 5 weeks has been suggested, since this significantly increases the antibody responses in lymphoma patients. For children with malignancy, immunization after at least 1 month off chemotherapy and when the peripheral leukocyte count exceeds 1,000 improves immunogenicity. Immunization within the first 6 to 12 months following bone marrow transplantation is ineffective, but responsiveness gradually returns by 2 years in most patients.

Immunization is generally safe in solid-organ transplantation, but many recipients of such transplants, including

heart, lung, and liver transplants, have deficient HAI responses (351, 353). A booster dose 1 month after the initial one does not generally improve response rates but may in some transplant patients. Antiviral chemoprophylaxis (discussed below) offers an alternative or supplemental means of protection.

Pandemic Candidate Vaccines

A wide range of candidate A(H5N1) vaccines have been tested in humans (354). The H5 HA is a weak human immunogen, and two doses of nonadjuvanted, inactivated split-vaccine with a high HA-antigen content (45 to 90 μg) are needed for immunogenicity in the majority of healthy adults and children (355). In 2007 this vaccine was approved by the FDA for use in first responders and individuals at risk for A(H5N1) infection, becoming the first avian influenza vaccine licensed in the United States. Alum adjuvants do not improve immunogenicity much, but proprietary oil-in-water adjuvants (e.g., MF-59 and AS03) provide substantial HA antigen sparing and cross-clade immunogenicity (356, 357). Whole-virus A (H5N1) vaccines also appear to reduce antigen requirements and give broader immunogenicity. AS03-adjuvanted vaccine with HA doses as low as 3.8 ug elicit strain-specific neutralizing antibodies and cross-reactive antibodies to heterologous A(H5N1) viruses. AS03-adjuvanted A(H1N1)pdm09 vaccine was also more effective than a nonadjuvanted one in children <10 years of age (358). This vaccine (Prepandrix, GSK) has been approved in multiple countries, including in the United States since 2013, for use in persons 18 years of age and older, at increased risk of exposure to the influenza A(H5N1) virus, and is included in the USA national stockpile. In general antibody titers decline rapidly but can be boosted with additional vaccine doses; prime-boost strategies elicit more robust immune responses (354). One dose of a nonadjuvanted A (H3N2)v vaccine is immunogenic in healthy adults (359).

Inactivated A(H7N7) and A(H7N9) vaccines are also poorly immunogenic in humans, but two doses of AS03- or, to lesser extent, MF-59- adjuvanted inactivated A(H7N9) vaccine induces robust antibody responses in adults (360, 361).

Live Attenuated Vaccines

Intranasally administered live attenuated influenza virus vaccines (LAIV) have been used extensively in Russia and were licensed for commercial use in the United States in 2003. Cold-adapted, temperature-sensitive, attenuated donor viruses (A/Ann/6/60/H2N2 and B/Ann Arbor/1/66), which are able to grow at 25°C but are restricted at 39°C, have been used to make reassortants containing six internal genes from donor virus and two genes encoding the HA and NA from wild-type strains. Several gene segments contribute to the attenuation/temperature sensitivity phenotype of the live attenuated influenza A (with mutations in PB1, PB2, and NP) and influenza B (with mutations in PA, NP, and M1) virus vaccines (362, 363). These reassortant viruses are well tolerated, genetically stable, rarely transmissible to contacts, and immunogenic, following intranasal administration in seronegative children and adults. Serum antibody responses are lower in adults than those detected after administration of inactivated vaccines.

Immunogenicity

Immune responses to multivalent LAIV are influenced by preexisting immunity, the infectivity of each vaccine virus, and interference among vaccine viruses. The 50% infectious doses are about 10- to 100-fold lower for seronegative infants than for adults, but interference among vaccine strains reduces responses in seronegative children. Although single doses are often immunogenic (364), two doses appear to be necessary to confer multistrain protection in young children. Potential advantages of cold-adapted (ca) vaccines include ease of administration (nose drops or coarse spray), induction of local secretory antibody, protection against drift variants, and possibly induction of heterosubtypic CTL responses.

Effectiveness

The efficacy of LAIV in children aged 6 to 59 months is approximately 70 to 90% but has been negligible in some seasons (322, 364, 365). Its efficacy has been superior to that of inactivated vaccine in healthy children, including protection against antigenically drifted strains (366, 367), although not during recent seasons (365). As a consequence, the ACIP has recommended against use of LAIV in children for the 2016–17 season. LAIV efficacy is often lower than that of inactivated vaccine in healthy adults ≤65 years old (368–370), perhaps because of preexisting immunity inhibiting LAIV replication. In children, LAIV is protective against influenza-associated complications, including otitis media (364). School-based immunization programs can increase coverage, decrease influenza rates, in part through herd immunity, and improve school attendance (371). Wide-scale immunization of school-aged children appears to lessen the community impact of epidemics (372). Compared to inactivated vaccine alone, combined administration of inactivated and intranasal LAIV may have given greater protection against influenza in elderly nursing home residents in one trial (373), although not significantly so in ambulatory patients with chronic obstructive pulmonary disease (374).

Reactogenicity

LAIV is licensed for use in the United States only in healthy persons aged 2 to 49 years, but it appears to be safe in those with underlying pulmonary disease or HIV infection (CD4 count, >200 cells/mm^3) (Table 8). Intranasal LAIV may be associated with coryza (5 to 30% of recipients), sore throat (10%), and in children, transient febrile reactions (5%) or decreased activity level, typically on the second or third day after inoculation. An increased frequency of medically significant wheezing episodes has been found in children younger than 2 years of age (367). Detection of LAIV virus lasts up to 1 week in adults and up to 3 weeks in very young children. Transmission of vaccine virus may sometimes occur among young children. Healthcare workers and others who receive LAIV should avoid contact with highly immunocompromised persons (e.g., stem cell transplant recipients in protected environments) for 1 week after immunization. Whether LAIV might be associated rarely with GBS requires further study (375). LAIV appears to be safe in children aged 2–18 years with a history of egg allergy (499).

Pandemic Candidate Vaccines

Candidate A(H5) LAIV vaccines, 6:2 reassortants containing the HA and NA genes of an influenza virus of pandemic potential, have shown variable immunogenicity. One with an Ann Arbor backbone had very limited replication and antibody responses in volunteers, despite high intranasal doses (376). However, this vaccine induces long-lasting immunity that leads to cross-clade immunogenicity after boosting with inactivated vaccine (377). Another LAIV using the A/Leningrad/134/17/57 ca/ts virus backbone and an HA derived from low pathogenicity A/duck/

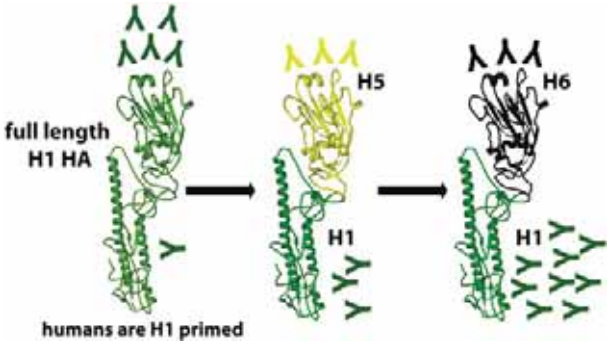

FIGURE 13 Chimeric hemagglutinin-based universal influenza virus vaccine approach. Most humans have preexisting immunity against the H1 hemagglutinin (HA) (green). The majority of the antibodies is directed against the immunodominant head of the HA (green) because of repeated exposure to H1N1 viruses. Few antibodies (green) are made against the immunosubdominant stalk of the HA. Upon vaccination with a chimeric HA vaccine containing, for example, a cH5/1 HA, the HA stalk antibodies (green) will be boosted, but only a primary (low) response against the novel H5 head (yellow) will be measured. A second boost with another chimeric vaccine cH6/1, which expresses the same H1-stalk (green), will further enhance the stalk antibodies. The response to the H6 head domain is again a (low) primary response (black). For a complete universal influenza virus vaccine, two chimeric influenza A virus components are needed (one, expressing a group 1 HA stalk and the other, expressing a group 2 HA stalk) and a third one, which expresses an influenza B virus stalk (all in the context of exotic HA head domains). Such a vaccine with chimeric HAs should also result in enhanced protective anti-NA responses mediated by the (immunosubdominant) neuraminidases present in the preparation. Courtesy of Krammer reprinted with permission.

Potsdam/1402-6/86 (H5N2) virus (378) and has been licensed for use in Russia; it also is effective at priming responses to inactivated vaccine (379). LAIV vaccines have also been developed for H7 virus infection (380) and also prime for responses to inactivated vaccine (381). The potential for reassortment during co-infection with a LAIV pandemic vaccine and seasonal influenza viruses to yield transmissible, virulent strains constrains use of the former until a pandemic virus has emerged.

Novel and Universal Vaccines

Public health needs exist for more effective, long-lasting, and more broadly protective influenza vaccines. The first approach to make improved vaccines and to produce them faster would be to undertake efforts to eliminate the more than half-century-old manufacturing process using embryonated eggs for growth and utilize cell culture-based production. Most current vaccine production does not take advantage of reverse genetics techniques (382) (see Figure 4B) that would allow the rapid use of master seed viruses with defined high yielding characteristics and permit sequence-based quality controls of the vaccine products (383, 384) (Table 9). Further strategies involve the expression of recombinant proteins in a variety of cell systems including plant and insect cells (385–387) and the production of recombinant virus-like particles (VLP) in insect cells. An experimental A(H7N9) vaccine of this type combines the HA and the NA with an M1 protein (388).

In order to broaden the protective efficacy of influenza virus vaccines, different combinations of prime-boost vaccination strategies are being investigated with promising results (389). Whereas these approaches are not aimed at heterosubtypic immunity (against different subtypes), they are likely to provide better heterogeneous protection (against different variants of the same subtype) than currently available vaccines. Whether virus-vectored influenza virus vaccines will be successful remains uncertain because repeated vaccination with the vector is probably made more difficult due to initial immune responses against the vector itself. Such an approach may, however, be advantageous in inducing strong T cell-mediated responses (390, 391). Other approaches include live-attenuated vaccines based on either the truncation of the NS1 protein (392) or the complete deletion of the NS1 (393), PB2 (394), or HA (395, 396) proteins. The latter constructs induce potent T cell responses. Another approach is the use of novel constructs (31) that present B- and T-cell epitopes, which are combined into a cocktail (Multimeric-001) for the purpose of inducing broad immunity against A and B strains.

The development of a universal influenza virus vaccine providing long-lasting immunity against viruses expressing different HA and NA subtypes remains elusive. Early on, subviral particles, prepared by acid (pH5) and mercaptoethanol treatment of purified virus, induced a strong immune response in animals against the conserved HA2 domain of the HA. However, this vaccine construct did not protect against subsequent virus challenge (397), presumably because the epitopes inducing a protective response had been destroyed by the chemical treatment of the virus. More recently, the highly conserved 22-23 amino-acid ectodomain of the M2 transmembrane protein (M2e) has been used (in the form of fusion proteins) to induce cross-protective immune responses (398, 399), although these have yet to undergo field testing. Another concept of a universal influenza virus vaccine is based on "centralized sequences" or computationally optimized broadly reactive antigens (COBRAS) of HAs (400). Although the earlier attempt to construct a vaccine based on the conserved HA2 (in essence the conserved stalk domain) was not successful (397), an H1-based headless HA displayed on a virus-like particle showed cross-reactive antibodies against subtype 2 and subtype 5 HAs (401). Additional headless and peptide constructs comprising epitopes of the stalk region are being studied, but they show limited efficacy in mouse models as yet (402–405).

A more promising approach to expanding anti-HA stem responses appears to be the use of vaccines containing a chimeric HA. In the simplest application, individuals who have been exposed to H1N1 viruses would be immunized once with vaccine expressing a chimeric HA (e.g. cH5/1), which has an exotic H5 head domain and the stalk of the H1 HA. This vaccination strategy would amplify the B memory cells directed against the stalk of the H1 HA (and very few antibodies would be directed against the H5 head). A second immunization with a virus containing the chimeric cH6/1 HA (expressing an H6 head domain and the same H1 stalk) would result in further stimulation of stalk antibodies but a low response to the H6 head domain (Fig. 13). In effect, this immunization strategy represents a redirection of the immune response from the immunodominant head domain towards the HA stalk component of the vaccine (406, 407). It is hoped that this redirection towards the immunesubdominant HA stalk would enhance the induction of NA antibodies. The NA exhibits low antigenic drift and antibodies against the NA can be cross-protective in animal challenge models and are correlated with protection in

TABLE 9 Novel influenza virus vaccine technologies

Technology	Comments
Cell culture, reverse genetics and sequencing, synthetic vaccines	Rapid production, embryonated eggs not needed, improved quality control (382–384)
Recombinant protein expression in insect cells, plant cells, lactobacillus, algae, yeast	Rapid vaccine production, low production costs (385–387)
Virus-like particles expressed in mammalian, insect, or plant cells	Good safety and efficacy (385, 388, 482)
DNA LAIV, recombinant proteins, IIV combination booster vaccinations	Broader and stronger immune responses than vaccination with individual vaccine preparations (389, 482)
Virus vector vaccines	Potent humoral and cell-mediated responses (390, 483)
M2e (universal) fusion proteins and multimeric constructs	Promising approach to develop universal vaccine (398, 399)
Attenuated constructs, peptide vaccines	Induction of humoral as well as cellular immunity (392–396, 484)
Centralized (COBRA) HAs	Elicit broadly-reactive set of antibodies in animals (400)
Headless and chimeric HAs	Induce broadly protective antibodies to the conserved HA stalk domain and also allows the redirection of the immune response to the conserved NA protein (397, 401–407, 485)

humans (314). Thus, enhanced antibody responses to the conserved regions of influenza viruses (HA stalks and NAs) may be the key for a universal influenza virus vaccine. The influenza A virus HAs fall into two groups (Fig. 2); thus, a vaccine inducing protective responses to group 1 HA stalk and to the stalks of group 2 may be protective against viruses from all known HA subtypes. Although it is not known what the overall contributions of the NA antibody responses to protection are relative to those of the HAs, it is possible that there is sufficient cross-protection within the NA groups that an N1 and N2 NA (belonging to group 1 and group 2, respectively) will cover all the NAs of circulating influenza viruses. A trivalent universal influenza virus vaccine would be designed to enhance immune responses against the group 1 HA stalk, the group 2 HA stalk, as well as the B virus HA stalk. At the same time, the trivalent vaccine would contain a group 1 NA (N1), a group 2 NA (N2), and a B virus NA (385). Such a vaccine (on different available platforms, plus or minus adjuvants), might provide universal protection against all influenza A and B viruses is to be tested in human trials.

Antiviral Chemoprophylaxis

The neuraminidase inhibitors (NAIs), inhaled zanamivir, oral oseltamivir, and inhaled laninamivir, are effective for prophylaxis for both influenza A and B virus infections (408, 409). Amantadine and rimantadine are effective for prophylaxis and treatment of influenza A virus infections due to susceptible strains (410), but neither is effective for influenza B, and recently circulating influenza A viruses are resistant (411). Oseltamivir and inhaled zanamivir are approved in the United States and many other countries for prophylaxis. Neuraminidase inhibitors have been stockpiled by WHO for both treatment and possible use in mass-targeted chemoprophylaxis for containing emergence of a pandemic virus (412).

Effectiveness

The efficacy of oseltamivir at 75 mg once daily for 6 weeks for seasonal (preexposure) prophylaxis is approximately 84% in unimmunized working adults and 89% in immunized nursing home residents. Preexposure prophylaxis also protects high-risk immunocompromised hosts (413). When used for postexposure prophylaxis in family contacts, 7 to 10 days of once-daily oseltamivir provides 68 to 89% protection (408). Inhaled zanamivir at 10 mg once daily is also highly protective against influenza when used for seasonal (82 to 83% efficacy) or postexposure (82% efficacy) prophylaxis (408).

Prophylactic oral amantadine and rimantadine are 70 to 90% effective in preventing illness caused by susceptible strains of influenza A virus (410). With sensitive strains, postexposure prophylaxis with rimantadine or amantadine for 10 days was effective in protecting family contacts when index cases were not treated, but concurrent treatment of ill children may lead to rapid selection and spread of drug-resistant virus to contacts and cause prophylaxis failures (410). In contrast, use of inhaled zanamivir or oral oseltamivir, both for treatment of ill index cases and for prophylaxis of contacts, is protective and usually not associated with resistance transmission. In one nursing home-based trial, zanamivir prophylaxis was more effective than oral rimantadine in protecting against influenza, in part, because of the frequent failure of rimantadine prophylaxis due to drug-resistant strains (414).

Seasonal prophylaxis is an alternative or adjunct to immunization when the epidemic strain differs antigenically to a significant extent from the vaccine strain or when high-risk patients have a contraindication or are not expected to mount an adequate response to immunization. Combined use of inactivated vaccine and chemoprophylaxis offers the highest level of protection for high-risk patients. Protection requires drug administration for the duration of the epidemic, generally 6 to 10 weeks; inhaled zanamivir and oral oseltamivir have been well-tolerated in healthcare workers up to 16 weeks (415). Drug recipients may experience subclinical infection, which usually confers protection against reinfection by the same strain.

Because antiviral chemoprophylaxis does not interfere with the immune response to inactivated vaccine, they can be administered concurrently. However, concurrent use of any anti-influenza antiviral drug might interfere with the

immunogenicity of LAIV. If influenza has already occurred in the community, prophylaxis can be given to unimmunized high-risk persons for 2 weeks beginning at the time of immunization. For likely exposure, NAI chemoprophylaxis is a consideration for unvaccinated persons, including pregnant women, at increased risk of complications. Inhaled zanamivir may be the preferred prophylaxis agent for pregnant women.

Management of Nosocomial Outbreaks

Prevention of outbreaks in institutional populations requires an established plan that includes preapproved vaccine and medication orders; preseason immunization of residents and staff members, surveillance for influenza during the season, restriction from work of employees with possible influenza illness, and, in the event of an outbreak, use of appropriate isolation techniques, immunization of residents and staff who have not received vaccine, and often antiviral chemoprophylaxis (416, 417). Chemoprophylaxis both for patients, irrespective of immunization status, and often for staff, is indicated for outbreak control. A policy of oseltamivir-treatment of ill persons and prophylaxis for residents and staff appears effective during influenza outbreaks in aged care facilities (418, 419). Administration for 2 weeks, or until new cases have ceased to occur for at least 1 week, appears to be adequate in most nursing home outbreaks. Currently NA inhibitors should be used for chemoprophylaxis since circulating influenza A strains are resistant to M2 inhibitors, and, even when outbreak strains are susceptible, resistance emergence and transmission is problematic with M2 inhibitors (414). However, nosocomial transmission of oseltamivir-resistant A(H1N1)pdm09 virus has sometimes caused lethal illnesses within hematology-oncology units (420, 421). Failures of oseltamivir prophylaxis, particularly in immunocompromised hosts, should prompt investigation for resistance emergence and other pathogens, as well as consideration of zanamivir use.

Whenever possible, infected patients should be isolated using standard and droplet precautions. In the context of nursing home outbreaks, efforts to reduce transmission include confinement of ill residents to their rooms for at least 72 hours; restriction of movement of nonsick residents to other parts of the facility and consideration of confinement to their rooms, if an outbreak with high attack rates or severe illness is occurring; decentralization or postponement of activities, which could expose large numbers of nonsick residents to influenza; minimizing work assignments of staff to multiple units of the facility; restricting visits by persons with respiratory illness; and discouraging visits to sick residents.

TREATMENT

Supportive Care

Symptomatic treatment of influenza commonly involves antipyretic-analgesic drugs, particularly acetaminophen or nonsteroidal anti-inflammatory agents, for relief of fever, aches, and other systemic symptoms. Aspirin should be avoided in children because of its association with Reye's syndrome. Increased mortality with aspirin or paracetamol has been observed in some animal models of influenza (422), and high-dose salicylates were reported to be associated with increased mortality during the 1918 pandemic (423). Maternal hyperthermia during the first trimester increases the risk of neural tube defects and possibly other birth defects, and maternal fever during labor has been shown to be a risk factor for adverse neonatal and developmental outcomes. Acetaminophen appears to be the best option for treatment of fever during pregnancy.

Antitussives are often needed for relief of cough. Antibiotics have not been shown to benefit the course or reduce the likelihood of complications and should be used for proven or presumed bacterial complications. In those with lower-airway disease, correction of hypoxemia and treatment of bronchospasm are important. Advanced mechanical ventilation techniques (low tidal volumes, judicious use of positive end-expiratory pressure [PEEP]) and increasing use of extracorporeal membrane oxygenation (ECMO) have improved the outlook in severe cases, but case fatality remains high in influenza-associated ARDS.

Immunomodulators

Because host responses contribute to influenza disease pathogenesis, anti-inflammatory and immunomodulatory agents are important considerations for therapeutic intervention (424). Systemic corticosteroids have been given commonly to patients with influenza-associated pneumonia and ARDS without evidence of benefit. Retrospective studies of patients with A(H1N1)pdm09 or avian influenza infections indicate an association between corticosteroid use and increased risks of complications, including nosocomial pneumonia and invasive fungal infections, possibly increased mortality, and prolonged viral replication and emergence of antiviral resistance (121, 146, 425–427). Consequently, corticosteroid treatment of influenza patients should be avoided unless clinically indicated for other reasons (e.g., exacerbation of reactive airways disease, adrenal insufficiency, low-dose hydrocortisone for refractory septic shock). The value of corticosteroids to treat bronchiolitis obliterans with organizing pneumonia or the fibroproliferative phase of ARDS associated with viral pneumonia is uncertain (428). Pulse methylprednisolone is part of the commonly used treatment regimen (combined with antivirals and intravenous immunoglobulin) for treating influenza-associated encephalopathy in Japan.

A wide variety of immunomodulatory agents, most directed against excessive proinflammatory host responses, show benefits in animal models of influenza (e.g., gemfibrizol, pioglitazone, cyclo-oxygenase 2 inhibitors, pamidronate, N-acetylcysteine, erythromycin, resveratrol) (426, 429), but clinical data are limited or absent. During severe influenza A virus infection in mice, platelet activation worsens acute lung injury, and anti-platelet therapies diminish its severity (430). The use of widely available, low-cost interventions like HMG-CoA reductase inhibitors (statins) has generated considerable interest (431). While retrospective studies have reported mortality reductions in patients receiving statins, who were subsequently hospitalized for influenza (432) or pneumonia, findings are negative in some animal models (433, 434) and clinical studies (435). Large randomized controlled trials (RCTs) testing statins as therapy for ARDS due to sepsis or other causes have not shown clinical benefit (436, 437). Further clinical studies are needed to determine the possible benefits and optimal timing of use for particular host-directed therapies.

Antivirals

The antiviral spectra, pharmacology, effectiveness, tolerability and safety, and dosing regimens of approved influenza antivirals are discussed in Chapter 14. In general, antiviral treatment is warranted as soon as possible for patients with

suspected or confirmed influenza who have severe, complicated, or progressive illness, and in those requiring hospitalization (221). Treatment is also recommended as soon as possible for outpatients who are at higher risk for influenza complications on the basis of their age or underlying medical conditions (Table 7), depending on their clinical circumstances. Early antiviral treatment (within 2 days of symptom onset) can also benefit previously healthy persons those with apparently uncomplicated illness.

Amantadine and Rimantadine

Currently, the use of M2 inhibitors is not recommended because of widespread resistance among circulating influenza A strains (221). Resistance due to the Ser31Asn substitution in M2, sometimes in association with other M2 changes, is present globally among strains of A(H3N2) and A(H1N1) pdm09 viruses and is present in all A(H7N9) and clade 1 and some clade 2 A(H5N1) viruses (221, 496). During therapeutic use in infections, due to susceptible strains, viruses cross-resistant to amantadine and rimantadine have been recovered from patients as early as 2 to 3 days after starting treatment and from about 30% of outpatient children and adults treated for influenza. Resistant variants are fully transmissible and cause typical influenza illness and associated complications (410).

In uncomplicated influenza A due to susceptible strains, early treatment (within ≤48 h of illness onset) with amantadine or rimantadine reduces viral replication and the duration of fever, symptoms, and functional disability by about 1 to 2 days in previously healthy adults. Treatment is associated with more rapid improvement in peripheral airway dysfunction but not airway hyperreactivity. Whether treatment reduces the risk of influenza-related complications or provides therapeutic benefit in established complications or severe influenza in hospitalized patients is unknown. In children, rimantadine treatment has been associated with lower symptom burden and viral titers during the first 2 days of treatment than with acetaminophen but also with more prolonged shedding of virus and emergence of resistance. Rimantadine is not approved for therapy of influenza in children in the United States.

Zanamivir

Inhaled zanamivir treatment provides 1- to 2.5-day reductions in time to alleviation of illness and return to usual activities compared to placebo in adults and children ≥5 years old, including those with mild to moderate underlying reactive airway disease, with uncomplicated influenza (497). Zanamivir appears to be more effective than oseltamivir in treating influenza B virus infections in children (438). Zanamivir treatment reduces the frequency of antibiotic prescriptions for clinically diagnosed lower respiratory complications by about 40% (495). Among older adults, including those developing influenza despite immunization, inhaled zanamivir treatment appears to have therapeutic effects and tolerance similar to those in younger adults. Whether treatment of ill persons reduces the risk of virus transmission to contacts remains uncertain (439, 440). The effectiveness and safety of inhaled zanamivir in treating influenza-associated pneumonia is unproven, and effective lung delivery is likely problematic in those with serious disease (441). Attempted delivery of the lactose-containing commercial formulation in intubated patients has been complicated by blockage of ventilator filters and fatal outcome (442); this formulation is not appropriate for use in nebulizers or for mechanical ventilation. Inhaled zanamivir is generally well tolerated during therapeutic use, although rare cases of bronchospasm and exacerbations of underlying airway disease, sometimes fatal, have been reported, and the drug is contraindicated in those with significant airways disease. The breath-activated proprietary Diskhaler device for delivery of inhaled zanamivir requires a cooperative patient who can effectively inspire, and certain groups (children <5 years old, persons with cognitive impairment, and very frail or hospitalized elderly persons) may not able to use it reliably.

Zanamivir is inhibitory for most, although not all, oseltamivir-resistant variants, and intravenous delivery has been used extensively for treating influenza in seriously ill or immunocompromised patients with suspected or proven oseltamivir-resistant influenza infections (492). Its use in critically ill patients has been associated with acceptable tolerability and apparent antiviral effects (443). However, a phase 3 RCT did not find superiority to oral oseltamivir in treating adults hospitalized with influenza (444). Although uncommon, substitutions at residue Glu119 to Gly or Asp can confer resistance to zanamivir and other NA inhibitors in influenza A(H1N1)pdm09 viruses, particularly in combination with His275Tyr, and can cause therapeutic failure in immunocompromised patients (445, 446).

Oseltamivir

Early oseltamivir treatment of previously healthy adults with apparently uncomplicated influenza reduces the time to illness alleviation by approximately 1 to 1.5 days, time to resumption of usual activities by 2 to 3 days, frequency of lower respiratory secondary complications leading to antibiotic prescriptions, and all-cause hospitalizations by approximately 50% (447). The magnitude of clinical benefit relates to timing of initiation and is greater with therapy started within 12 hours of onset of symptoms (448). Oseltamivir treatment of children, aged 1 to 12 years with acute influenza, reduces illness duration by 1.5 days and the frequency of complications, particularly otitis media, leading to antibiotic prescriptions. Oseltamivir therapy appears to reduce the risk of progression to pneumonia in highly immunocompromised hosts with influenza and to reduce the risk of death in patients hospitalized with influenza, including pregnant women (240, 449, 450). Timely oseltamivir treatment is also associated with lower mortality in severe avian influenza virus disease, although progressive illness, sometimes related to resistance emergence, occurs (121, 145, 146, 502).

The safety of neuraminidase inhibitors in pregnancy have not been established, but no teratogenicity has been documented to date. Both zanamivir and oseltamivir carboxylate distribute across the placenta and into breast milk in animals. Given antiviral resistance patterns and the potential teratogenicity of the M2 inhibitors, the NA inhibitors are preferred for influenza treatment; the lower systemic exposure of inhaled zanamivir is a consideration in this decision.

Oseltamivir-resistant variants have been recovered from immunocompetent outpatient adults (∽1%), outpatient children (∽4%), inpatient children (∽18%), and subtype A (H5N1)-infected persons (∽25%) during or immediately after treatment (496). These variants typically possess amino acid substitutions (primarily Arg292Lys or Glu119Lys in N2 and His275Tyr in N1) in the NA. Oseltamivir-resistant seasonal A(H1N1) virus, harboring His275Tyr and other enabling NA substitutions (451), replaced susceptible virus and circulated globally before being replaced by the

A(H1N1)pdm09 virus. Since 2011 influenza A(H1N1) pdm09 viruses have also acquired NA substitutions that balance the adverse effects of H275Y on NA activity and surface expression. Temporally this has coincided with increasing evidence for community transmission of oseltamivir-resistant influenza A(H1N1)pdm09 H275Y viruses (452). Prolonged replication with resistant variants, usually with dual resistance to M2 inhibitors and sometimes with resistance to zanamivir, has been recognized in immunocompromised hosts (453, 496). Transmission of oseltamivir-resistant variants has been observed in healthcare settings and in the community (420, 421, 454). Intravenous zanamivir, available on compassionate use basis, is currently the most reliably active single agent for treating oseltamivir-resistant infections (455).

Peramivir

Single-dose intravenous peramivir is comparable in antiviral and clinical effects to a standard 5-day course of oral oseltamivir in uncomplicated influenza, although it is no better than oseltamivir for infections due to oseltamivir-resistant A (H1N1) virus (456). Treatment within 24 hours of symptom onset is associated with a shorter clinical course compared to placebo (457). Multiple dose regimens have been used in hospitalized patients, including those with severe viral pneumonia, but one RCT in hospitalized children and adults did not confirm more rapid clinical improvement (458). Peramivir has reduced inhibitory effects for many oseltamivir-resistant variants, including viruses containing His275Tyr in N1 or Arg292Lys in N2 and N9, and cannot be used reliably in such infections (146, 456). While no parenteral antiviral is currently FDA-approved for influenza management in seriously ill patients, intravenous peramivir is available in some countries (Japan, South Korea, United States, China).

Laninamivir

Single doses of the long-acting, orally inhaled NA inhibitor laninamivir are reported comparable in effectiveness to oral oseltamivir for treating uncomplicated influenza in adults (459) and to inhaled zanamivir in children aged 3 to 15 years (460). Laninamivir, currently approved only in Japan, has an antiviral spectrum similar to zanamivir and appears effective in treating oseltamivir-resistant A(H1N1) infection in children aged 3 to 9 years (461), although not in adults (459). However, an international RCT testing a higher dosage failed to show more rapid clinical recovery compared to placebo (462).

Favipiravir

Favipiravir (also designated T-705) is an influenza polymerase inhibitor active against all three influenza types, including variants resistant to M2 and NAIs, and some other RNA viruses in animal models (463). It has been approved in Japan but is under government control for potential use only when there is an outbreak of novel or reemerging influenza virus infections in which other anti-influenza virus agents are not effective or insufficiently effective, in part because of its teratogenicity in preclinical studies and contraindication for use in pregnant women. A twice-daily regimen showed significantly better antiviral and clinical effects than placebo in uncomplicated influenza (464), and the results of recently completed RCTs in outpatient adults are awaited. Favipiravir shows enhanced antiviral effects in combination with NAIs in preclinical models (465), but combination studies have not been undertaken as yet in severe influenza.

Investigational Agents

A number of anti-influenza compounds and combinations of antivirals or antivirals and immunomodulators have been described in preclinical studies, but few have undergone clinical testing (426, 465–467). One retrospective analysis suggested that therapeutic use of convalescent-phase blood products in pneumonia patients in 1918 reduced mortality (468), and early serotherapy with convalescent plasma or hyperimmune globulin-containing higher-neutralizing antibody titers, in combination with NA inhibitors in recent reports, likely reduces mortality in seriously ill patients (469, 470). A number of heterosubtypic, neutralizing antistem monoclonal antibodies, targeting HA group 1 and/or group 2, show therapeutic activity in animal models of lethal influenza including A(H5N1) and A(H7N9) viruses (471–473), and several of these and other antibody preparations (474) are in clinical trials.

Older investigational agents include ribavirin, a nucleoside analog active in preclinical models against influenza A and B viruses. If initiated early after symptom onset, aerosolized ribavirin variably reduces illness in adults with uncomplicated influenza, but it was associated with no important clinical benefits in young children hospitalized with influenza (475). Aerosolized, oral, and intravenous forms have been used to treat individual patients with influenza pneumonia or other severe complications, but ribavirin remains an investigational agent of unproven efficacy in influenza. Intranasal recombinant IFN-*a*2 partially protects against illness in experimental human influenza but is ineffective in preventing natural influenza (51).

Other novel inhibitors under clinical study include the oral PB2 inhibitor V-787 (476), the inhaled sialidase DAS181 (477), nitazoxanide (478), and the HA inhibitor arbidol (479). Various combinations of antivirals immunotherapeutics show enhanced antiviral activity in preclinical studies, including lethal avian influenza virus models, and warrant testing in severe infections in humans (465, 471). Ribavirin shows enhanced antiviral activity when combined with M2 or NAIs for dually susceptible strains under experimental conditions (465). A triple-drug regimen of amantadine, oseltamivir, and ribavirin, which is inhibitory for variants resistant to single drugs, has been used in critically ill and immunocompromised patients (480, 481) and is undergoing further clinical testing in at-risk outpatients.

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Bunyaviridae: Orthobunyaviruses, Phleboviruses, Nairoviruses, and Hantaviruses

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The family *Bunyaviridae* is the largest family of viruses and includes many known human, animal, and plant pathogens. The clinical diseases produced in humans range from acute febrile illnesses, such as sandfly fever, to more distinct clinical syndromes such as California encephalitis (CE), Rift Valley fever (RVF), Crimean-Congo hemorrhagic fever (CCHF), hemorrhagic fever with renal syndrome (HFRS), and hantavirus cardiopulmonary syndrome (HCPS), which is also referred to in the literature as hantavirus pulmonary syndrome (HPS). Sandfly fever, RVF, and HFRS are common. Although most of the remaining diseases probably cause no more than a few hundred cases each year, some are associated with a high mortality rate (particularly CCHF and HCPS), and two (CE and HCPS) are endemic in North America.

New and emerging viruses are still recognized and included in the large and growing family of *Bunyaviridae*. A new tick-borne phlebovirus that emerged in China during 2007 to 2009 has caused more than 2,500 cases of "severe fever with thrombocytopenia syndrome" (SFTS) (1), and, a new genetically related phlebovirus, the Heartland virus, was detected in two farmers with severe febrile illness and thrombocytopenia in Missouri in 2009 (2). Heartland virus is now recognized in 15 states. Heartland virus is spread by *Amblyomma americanum* (lone star tick) and may also infect wildlife and domestic animals, which could serve as reservoirs (3–5). Thus, clinicians should be aware that in undiagnosed febrile and severely ill patients with thrombocytopenia it is important to collect blood and other specimens for analyses.

VIROLOGY

Classification

The family *Bunyaviridae* includes more than 300 viruses and is divided into five genera: Orthobunyavirus, Phlebovirus, Nairovirus, Hantavirus, and Tospovirus (6). All genera except the Tospoviruses, which are plant viruses, infect vertebrate hosts and include human pathogens.

Representative groups and complexes for the orthobunyaviruses, phleboviruses, and nairoviruses, including major human pathogens, are shown in Table 1. In general, the genotypes are determined by molecular features, including conserved nucleotide sequences, genomic size, and genomic organization, whereas groups within each genus are determined primarily by serologic methods (6, 7). Within the larger genera, viruses may be classified into groups and complexes.

Antibodies are directed against a nucleocapsid protein (NP) and two major envelope glycoproteins. The nucleocapsid protein is more conserved within a genus than the glycoproteins. Serologic tests that are solely or primarily directed against nucleocapsid protein antigens tend to be sensitive but exhibit significant cross reactivity, whereas assays that are directed solely or primarily against glycoprotein antigens tend to be useful in distinguishing between closely related viruses (6, 7).

Virion Structure, Genome Composition, and Major Proteins

Bunyaviruses are enveloped viruses about 100 nm in diameter (80 to 120 nm) with 5 to 10 nm glycoprotein spikes projecting from the envelope (Figure 1) (8–10). The virion structures are known for several members of the *Bunyaviridae*, and the structures of the spike proteins on the surface vary considerably across genera (11–14). The viruses are generally spherical but may be oval and elongated. Virion size may vary, and by cryoelectron microscopy (Cryo-EM) and Cryo-EM tomography Hantaan virus (HTNV) particles range in size from 120 to 154 nm (12). The envelope surrounds a tripartite ribonucleoprotein complex containing the small (S), medium (M), and large (L) genome segments of single-stranded RNA with negative polarity (Figure 1).

The lengths of the genomic segments vary among the genera, ranging from 6.3 to 12 kb for the L segment, 3.5 to 6 kb for the M segment, and 1 to 2.2 kb for the S segment. The range within each genus is much more restricted. For hantaviruses, segment lengths range from 6.5 to 7.0 kb for the L segment, 3.6 to 3.7 kb for the M segment, and 1.6 to 2.1 kb for the S segment.

Four major structural proteins are encoded by the three segments, and nonstructural proteins are coded by some but not all viruses within the family. RNA-dependent RNA polymerase (RdRp or L protein) is coded by the L segment, nucleocapsid (N) protein by the S segment, and glycoproteins by the M segment (Figure 1). Many of the structural

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TABLE 1 *Bunyaviridae*: Genera, subgroups, and selected viruses that are human pathogens, excluding hantaviruses

Genus	Serogroup or complex	Geographic location of human pathogens	Selected human pathogens
Orthobunyavirus	California	North America, Europe	California encephalitis, La Crosse, Jamestown Canyon, Tahyna
	Simbu	South America	Oropouche
	Anopheles A	South America	Tacaiuma
	Bunyawera	Africa, South America	Bunyamwera, Fort Sherman, Shokwe, Xingu
	Group C	North and South America	Apue, Itaqui, Madrid, Oriboca
Nairovirus	Crimean Congo Hemorrhagic Fever (CCHF)	Africa, Asia, Europe	CCHF and Dugbe
	Nairobi sheep disease	Africa, Asia	Nairobi sheep disease
Phlebovirus	Severe fever with thrombocytopenia syndrome	Asia, North and South America, Africa,	Severe fever with thrombocytopenia syndrome
	Sandfly fever group	Europe, Asia	Alenquer, Punta Toro, Rift Valley fever, Sandfly fever Naples, Toscana, Chagres, Sandfly fever Sicilian

proteins for the major viruses in the family have been sequenced (15–19), and successful reverse genetics systems have been described (20–23).

Replication Strategies

Large gaps exist in understanding the mechanisms for Bunyavirus entry and assembly. As an example, Hantavirus virions are generally asymmetric, pleomorphic particles; the virion has a surface structure composed of an unusual square, grid-like pattern distinct from other genera in the *Bunyaviridae* and a lack of icosahedral symmetry typical of most viruses. The square spike on the outer surface reflects the glycoprotein (GP) projections, which extend ∽12 nm from the lipid bilayer and comprise 4 molecules of Gn and Gc (12). Hantaviruses bind epithelial and endothelial cells via interaction of Gn with the host's cell surface receptor(s); β1 integrin for apathogenic and β3 integrin for pathogenic hantaviruses (24, 25); although these may not be the sole receptors (26). HTNV enter through clathrin-coated pits, followed by movement to early endosomes, and subsequent delivery to late endosomes or lysosomes (27, 28).

Internally, the virion contains three rod-like ribonucleoprotein (RNP) structures, which presumably contain the viral RNA genome wrapped in N proteins. The viral RNA dependent RNA polymerase (RdRp) or L protein would be expected to be part of the RNP. For hantaviruses, the N protein is the most abundant viral protein synthesized early in infection and likely plays key roles in several important steps in the virus replication cycle (27) including assembly into the RNP and packaging. It is highly likely that different oligomeric states or conformations of N occur during the life cycle. The HTNV N protein traffics via microtubules in the cell to the ER-Golgi intermediate compartment (ERGIC) prior to viral assembly (28). It is unclear where or how the assembly of the RNP takes place; however, the RNPs must traffic to the Golgi since this is the compartment where virions have been visualized. The RNP presumably buds into the Golgi to produce the virion, and then the virion exits the Golgi through the formation of a vesicle, which would surround the hantavirus particles.

There is also considerable diversity in the replication strategies within the family, although all members of the family replicate in the cytoplasm and mature in the Golgi (Figure 1) (8–10, 29, 30). Attachment and entry are mediated by one or both of the glycoproteins (Figure 1). Phleboviruses enter cells by endocytosis, a mechanism that may be shared by other members of the family. *Bunyaviridae* can fuse cells at acid pH, and conformational changes in G1 have been described in the *Orthobunyavirus* genus. Thus, for members of the family, entry and uncoating may occur through a common mechanism of entry by endocytosis followed by uncoating and release of the three nucleocapsids into the cytoplasm following fusion of the viral envelope and endosomal membrane at acid pH.

After uncoating, primary transcription of the negative-sense vRNA to mRNA minimally requires vRNA, host primers derived from mRNA, and the viral polymerase (RDRP). The N protein is always associated with genomic RNA, so this protein may also be required. Transcription is followed by translation and trafficking of the RDRP and N proteins to the perinuclear area. This is followed by genome replication, usually through synthesis of a full-length positive-strand cRNA that serves as a replicative intermediate, followed by synthesis of negative-strand vRNA. These events are followed by continued translation and RNA replication.

Replication of the S segment in the *Phlebovirus* and *Tospovirus* genomes is accomplished via an ambisense coding strategy. The NS protein is encoded in the 5′ half of the S segment, the N protein is encoded in the 3′ half of the segment, and the proteins are translated from separate mRNAs. Genetic reassortment has been demonstrated both in cell culture and *in vivo* in arthropod vectors between members of the same serogroup in the genus *Orthobunyavirus*, but not between members of different serogroups in the same genus.

A key point in genome replication is the switch from transcription of genomic RNA to production of the positive-strand cRNA that serves as the replicative intermediate. This is also a key component in the transcription and replication strategies of other negative strand viruses such as vesicular stomatitis virus (VSV), influenza virus, and rhabdovirus. For VSV, the switch appears to be regulated by the N protein, and a similar mechanism may exist for members of the family *Bunyaviridae*.

These events are followed by terminal glycosylation of the glycoproteins, assembly of viral particles by budding into Golgi vesicles, transport of the cytoplasmic vesicles to the cell surface, fusion of the cytoplasmic vesicles with the plasma membrane, and release of virions through exocytosis.

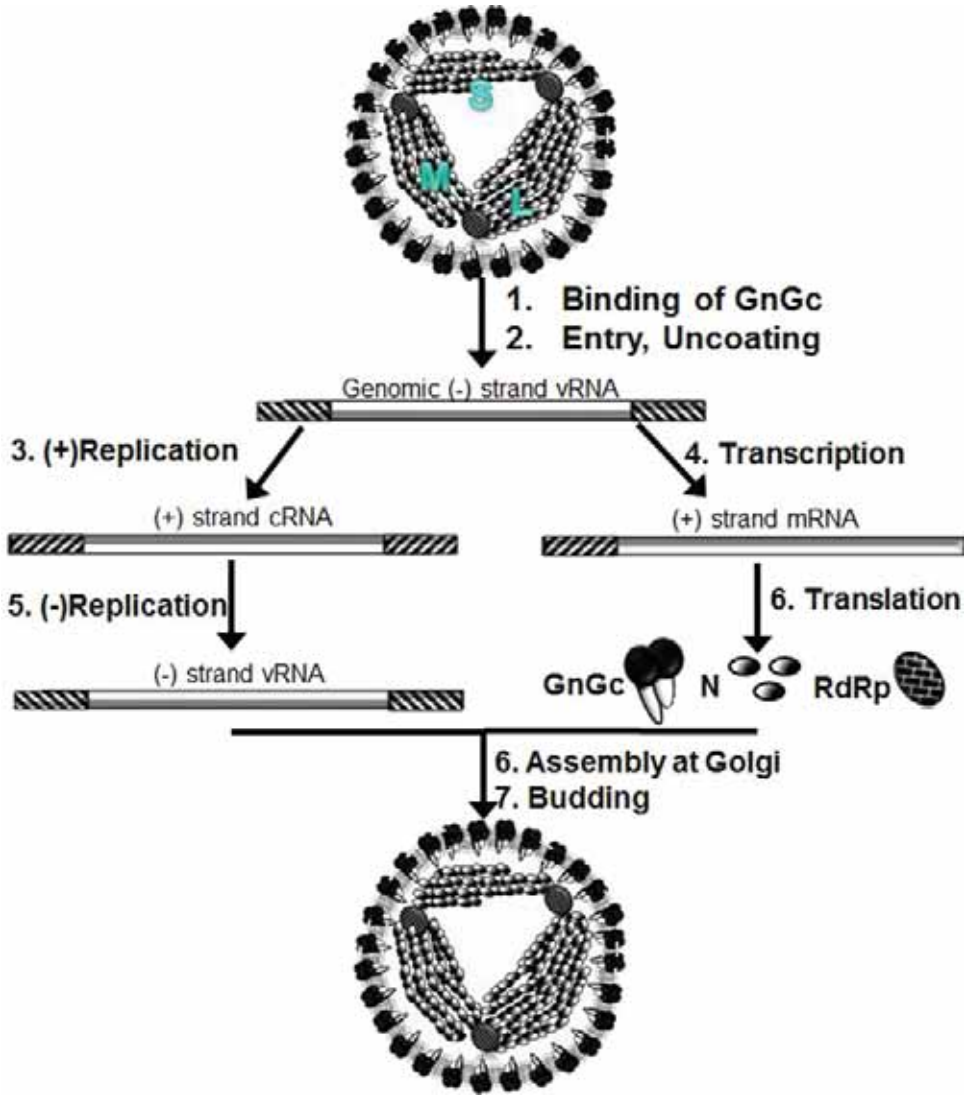


FIGURE 1 Model for *Bunyaviridae* replication. The principal steps in replication are (1) attachment of Gn to integrin receptor; (2) entry and uncoating; (3) replication, cRNA synthesis from vRNA genome; (4) transcription, mRNA synthesis from vRNA genome; (5) replication, vRNA synthesis from cRNA template; (6) translation, L and M mRNA translated on free ribosomes and M translation on RER; N and RdRp and L, M, and S genomes move to Golgi; (7) packaging, assembly of genomic RNAs and proteins at Golgi (some evidence for New World viruses assembling on plasma membrane); and (8) budding. Virion release, Golgi vesicles release virion by budding with plasma membrane.

Unlike other negative-strand RNA viruses. *Bunyaviridae* lack a matrix protein that bridges the gap between envelope proteins and the nucleocapsids during assembly. Thus, there may be direct transmembrane recognition between viral glycoproteins that accumulate on the luminal side and the ribonucleoprotein structures (ribonucleocapsids) that accumulate on the cytoplasmic side of vesicular membranes. In addition to morphogenesis in the Golgi that occurs in all members of the family, additional mechanisms have been described. For example, one member of the genus *Phlebovirus*, RVF virus, can bud at the surface of rat hepatocytes as well as into the Golgi cisternae.

Host Range, Growth in Cell Culture, and Inactivation

Members of the *Orthobunyavirus*, *Phlebovirus*, and *Nairovirus* genera infect both vertebrates and arthropods. Given the large number of viruses in the family, it is not surprising that the overall range of natural vertebrate hosts is broad, including rodents, lagomorphs, deer, birds, and sheep and other domestic animals (6, 7). Humans are not a natural reservoir for any members of the family *Bunyaviridae*, but human infections, some of substantial medical importance, occur by members of each genus except the *Tospovirus* genus. However, for a particular virus, the range varies from broad to very limited, the latter being particularly true of hantaviruses. Although "spillover" may occur between small rodent species in nature and rats, mice, and rabbits can be infected in the laboratory, each hantavirus tends to have a very restricted host range in nature (Table 2). The limited host range and similar phylogenetic relationships among hantaviruses and among the rodent hosts have led to the suggestion that hantaviruses may have co-evolved with their rodent hosts. Recent evidence also suggests remote

TABLE 2 Hantavirus species recognized to date. Only those that are well characterized and clearly differentiated from previously characterized species are listed. Courtesy of Dr. B. Hjelle.

Virus	Abbreviation	Synonyms	Host	Distribution of host	Disease
Hantaan	HTN		*A. agrarius*	Central & E Asia, Central & E Europe	HFRS
Seoul	SEO		*R. norvegicus*, *R. rattus*	Worldwide; commensal rat hosts	HFRS, mild
Dobrava/Belgrade	DOB	BEL	*A. flavicollis*	Asia Minor, Europe, Palestine	HFRS, severe
Saarema	SAA		*A agrarius*	Central and East Europe	HFRS, mild
Puumala	PUU		*C. glareolus*	Russia, Europe, Asia Minor	HFRS/NE
Sin Nombre	SN	Four Corners	*P. maniculatus*	Throughout US, W Canada	HCPS
Black Creek Canal	BCC		*S. hispidus*	SE US to Peru	HCPS
New York	NY	NY-1	*P. leucopus*	NE US, SE Canada	HCPS
Bayou	BAY		*O. palustris*	SE US, Kansas to New Jersey	HCPS
Andes[a]	AND		*O. longicaudatus*	Southern Chile, Argentina	HCPS
Laguna Negra	LN		*C. laucha*	Paraguay, Bolivia	HCPS
Choclo			*O. fulvenscens*	Panama	HFRS, mild
Thottapalayam	TPM		*S. murinus*	Africa, India, SE Asia	unknown
Tula	TUL		*M. arvalis*	Russia, Europe, Asia Minor	unknown
Thai	THAI		*B. indica*	SE Asia, India	unknown
Khabarovsk			*M. fortis*	East Asia	
Prospect Hill	PH		*M. pennsylvanicus*	N, E US, Canada, Alaska	unknown
Bloodland Lake	BLLL	PVV	*M. ochrogaster*	midwestern, E US, S Canada	unknown
Isla Vista	Isla	CMMV	*M. californicus*	California, Oregon, Mexico	unknown
El Moro Canyon	ELMC	HMV-1	*R. megalotis*	W US, Mexico, SW Canada	unknown
Rio Segundo	RIOS	HMV-2	*R. mexicanus?*	Mexico, Costa Rica, Ecuador	unknown
Rio Mamoré	RM		*Oligoryzomys microtis*	Bolivia, Brazil, Paraguay, Peru	unknown
Calabazo			*Z. brivicauda*	Panama	unknown

[a]Possibly synonymous with variant forms Bermejo, Oran, Lechiguana.

divergence of a clade of insectivore-borne hantaviruses (31, 32). Although many members of the latter clade have recently been identified in soricids (shrews), none have been clearly associated with human disease.

The range of arthropod hosts tends to be more limited within each genus and to be even more limited for groups or individual viruses within each genus (Table 3). With only a few exceptions, members of the genus *Orthobunyavirus* infect only mosquitoes. Most phleboviruses infect sandflies, but RVF virus can infect a wide range of arthropods and appears to be transmitted primarily by mosquitoes. The arthropod host range for nairoviruses appears to be largely limited to ticks, particularly those in the genus *Hyalomma* (33, 34).

Most bunyaviruses replicate in BHK-21 (hamster) or Vero E6 (monkey) cells, are cytolytic, and plaque efficiently. In contrast to most other Bunyaviruses, hantaviruses are fastidious in cell culture and are generally not cytopathic. They can only be grown in a few types of cells, particularly Vero E6 cells, and in experimental infections in rodents such as suckling or adult mice. Hantaan, Seoul, Puumala, Prospect Hill, and Sin Nombre Virus (SNV and Convict Creek isolates) were originally recovered from their usual rodent hosts, albeit with some difficulty (7, 35–40). Viral antigen can be detected by fluorescent antibody or other techniques, and plaque reduction assays are also possible in Vero E6 cells. Bunyaviruses in the *Orthobunyavirus* genus have been shown to cause persistent, noncytopathic infections in mosquito cell cultures. Suckling mice may also be used for isolation of many members of the family. Like other enveloped viruses, bunyaviruses are sensitive to acid pH, detergents, formalin, heat, and lipid solvents.

EPIDEMIOLOGY

General Principles

For members of the *Orthobunyavirus*, *Phlebovirus*, and *Nairovirus* genera, the infection is arthropod borne, although RVF may also be acquired by direct contact with infected animal carcasses or blood. Person-to-person transmission may also occur with CCHF, SFTSV, and with Andes virus (ANDV) infections, including nosocomial transmission of CCHF through contact with infected blood and person-to-person transmission of ANDV within households and other close contacts, as well as rare nosocomial transmission in Chile and Argentina (41).

For most bunyaviruses, the period of viremia in the vertebrate host is brief, suggesting that infection in vertebrates is generally more important for amplification than for viral perpetuation. In contrast, infection in the insect is persistent. For several viruses in these genera, virus perpetuation can be maintained solely in the insect host for prolonged periods through both transovarial and horizontal transmission. For example, La Crosse virus survives over the winter in mosquito eggs, and RVFV can survive in mosquito eggs for years during drought.

In contrast to the genera described above, hantaviruses have no arthropod vector, although infection and transovarial transmission in gamsid mites have been described (42). Virus perpetuation and amplification occur largely within a single rodent species that is chronically infected (Table 2). Infected rodents appear asymptomatic, but PUUV impairs overwinter survival in bank voles and SNV-infected juvenile deer mice have shorter survival than uninfected

TABLE 3 Natural vertebrate hosts, vectors, and mechanism of transmission for representative bunyaviruses that are human pathogens

Genus/virus	Vertebrate hosts	Vectors	Transmission
Orthobunyavirus			
La Crosse	Woodland rodents	Aedes triseriatus	Insect bite
Bunyamwera	Rodents, lagomorphs	Aedes species	Insect bite
Nairovirus			
CCHF	Herbivores, lagomorphs	*Hyalomma* ticks	Insect bites, infected animal tissues, person-to-person
Phlebovirus			
Rift Valley fever	Sheep/domestic animals	Mosquitoes	Insect bite, contact with blood/tissue of animals
Sandfly fever	Unknown	Phlebotomus species	Insect bite
Severe fever with thrombocytopenia syndrome	Domestic & wild animals	Ticks	Insect bite, contact with blood/tissue of animals
Hantavirus	Rodents (persistent)	None	Aerosolized rodent excreta, person-to-person (Andes)

ones (43). SNV-infected adult male deer mice and SEOV-infected male rats are more aggressive when compared to uninfected adult males. This enhanced aggressive behavior likely facilitates transmission, since wounding is the primary mode of horizontal transmission (44). There is no evidence for vertical transmission, and most evidence points to horizontal transmission following weaning and clearance of protective maternal antibodies (45). Virus is chronically shed in rodent excreta, and humans appear to acquire infection from aerosols from infected rodent excreta. The viability virus in rodent excreta is not known, but hantavirus remains viable for at least 12 days at room and much longer at lower temperature (46, 47). Finally, laboratory infections have been documented with many members of the family. In most cases, aerosol transmission has been the probable mode of transmission, but direct transmission of hantaviruses from contact with infected animals may also occur. Particular care, including BL-4 containment for some applications, should be taken with agents that carry a high risk of mortality, like CCHF virus and the hantaviruses that cause HCPS.

CALIFORNIA ENCEPHALITIS SEROGROUP VIRUSES (*BUNYAVIRUS* GENUS)

Epidemiology

The virus type or subtype, geographic location, and primary mosquito vector and vertebrate host or hosts for California serogroup viruses that cause human infection are shown in Table 4. The CE group is named for the first virus in this group isolated from humans with encephalitis in Kern County, California in 1943. Since then, however, this virus has not been associated with human disease, and La Crosse virus, first identified in 1960 from a fatal case of encephalitis, has accounted for most reported cases of CE (48).

California encephalitis is endemic in the Midwest. Most cases occur between July and September in children, particularly boys, between the ages of 6 months and 16 years with a peak incidence in children between the ages of 4 and 10. Most infections appear to be subclinical or result in a febrile syndrome without encephalitis (48). One survey in Minnesota estimated that one encephalitis case occurred for every 26 childhood La Crosse virus infections, while a large serosurvey in Indiana suggested that there might be more than 1,000 infections for every reported case of encephalitis in children less than 16 years old. Acquisition of La Crosse infection continues throughout life with about 20% of the population seropositive in endemic areas by the age of 60; presumably, adults who acquire infection are at little risk of developing encephalitis.

The primary vector is the mosquito *Aedes triseriatus*. The female *Ae triseriatus* is a daytime feeder that breeds in rainwater in tree holes or other small containers, including discarded tires, thereby allowing breeding and disease transmission in areas distant from the mosquito's usual forest habitat. The virus can overwinter by transovarial transmission where eggs deposited in the fall can survive the winter and hatch in the spring (49). Evidence suggests that infected female mosquitoes may hibernate during the winter and lay eggs in the spring. At that point, virus can be amplified through infecting small mammals, particularly chipmunks and squirrels, and through horizontal transmission through mating with male mosquitoes. Humans are infected through bites from chronically infected female mosquitoes. As with other infections in this genus, infections in the mosquito are chronic, whereas viremia in the primary vertebrate hosts and humans is limited.

Another California serogroup virus, Jamestown Canyon virus, is widely distributed in North America and may be amplified in the white-tailed deer. It is transmitted by

TABLE 4 California serogroup viruses

Virus	Geographic location	Vector	Host	Human infection
California encephalitis	Western US, Canada	*A. melanimon, A. dorsalis*	Rodents, rabbits	Very rare
La Crosse	Midwestern, Eastern US	*A. triseriatus*	Chipmunks, squirrels	Endemic
Tahyna	Europe	*A. vexans, C. annulata*	Domestic animals, rabbits	Endemic
Jamestown Canyon	North America	*A. sp., C. inornata*	White-tailed deer?	Uncommon

Culeseta inornata and *Aedes* species. In contrast to La Crosse virus, Jamestown Canyon virus more commonly causes encephalitis in adults compared to children.

Pathogenesis in Humans

The incubation period is approximately 3 to 7 days based on exposure histories and the incubation period in experimental animals. Virus has only rarely been isolated from brain tissue of persons dying from encephalitis; virus has not been recovered from blood, CSF, or throat or rectal swabs (50). The few reported autopsies and a brain biopsy showed cerebral edema, mild leptomeningitis, glial nodules, perivascular cuffing and rare, focal necrosis distributed in cortical gray matter of the temporal, parietal, and frontal lobes; basal nuclei; midbrain; and pons (50–52).

Antibodies against G1 neutralize virus, block fusion, and inhibit hemagglutination and presumably are important for virus clearance, recovery from infection, and prevention of reinfection. Passive administration of neutralizing antibody protects experimental animals, and development of specific immune responses is correlated with recovery from illness in humans and in clearance of viremia in animals (50).

Clinical Manifestations

At the onset of the illness, patients typically have fever (100%), headache (∽75%), malaise, and nausea and vomiting (50 to 65%) (6, 51, 53, 54). Within 1 to 3 days this is accompanied by meningeal signs (∽60%) and lethargy, with total resolution within 7 to 8 days. In the most severe form, fever and headache may begin abruptly with progression to seizures in 12 to 24 hours. Overall, approximately half of cases develop seizures, including some with status epilepticus. Approximately 10% develop coma. Most of the remainder develop disorientation or altered consciousness, but aseptic meningitis without altered consciousness is also described. Less commonly, hemiparesis, tremor, aphasia, ataxia, dysarthria, abnormal reflexes, and chorea have been reported. The case fatality rate is 1% or less, and the total duration of the illness rarely exceeds 10 to 14 days. Most persons recover without any obvious residua. However, persistent focal neurologic findings or learning difficulties have each been reported in about 2% of patients, and emotional lability is reported to persist in about 10%. Epilepsy persists in about 20% of those who experience seizures during the acute episode, and most persons tested within the first five years after an acute episode have an abnormal electroencephalogram (55).

Most children with CE have a peripheral white blood cell (WBC) count between 10,000 and 20,000 (median ∽16,000), but normal counts and counts as high as 30,000 have been reported (47, 51–54). The cerebrospinal fluid (CSF) is almost always abnormal at some point during the acute illness. The CSF white blood cell count is elevated (median ∽100, range 0 to 600 cells/mm^3) in at least 90% of cases, with a predominance of mononuclear cells in 60 to 85% of patients. The CSF glucose is normal and the protein concentration is usually normal, but in about 30% of patients the latter is minimally elevated (range 40 to 100 mg/dl).

In the age group at greatest risk for CE, the most common illnesses in the differential diagnosis include herpes simplex virus encephalitis (HSE), mumps, meningitis or encephalitis, West Nile virus, or enteroviral meningoencephalitis (51, 54). Mumps and enteroviral infections tend to occur during community-wide epidemics. Parotitis would be diagnostic of mumps and rash would suggest enteroviral infection, although rash is rarely reported in CE. Focal CNS findings, seizures, stupor, or coma would suggest CE or HSE. Absence of exposure to a rural, wooded area in the past two weeks, occurrence outside the months of June through September, or illness in a child less than one year or greater than 15 years of age would suggest a diagnosis other than CE. Bacterial meningitis might be suggested in the 30% of patients with a predominance of leukocytes in the CSF, but the clinical course, normal glucose, and negative Gram stain and cultures would rule out bacterial meningitis (51).

Laboratory Diagnosis

Although California serogroup viruses cause productive, cytotoxic infections and replicate in many cell lines, including Vero or BHK-21, repeated attempts to grow virus from throat swabs, blood, CSF, and stool have been unsuccessful. The diagnosis is usually established by serologic methods, including neutralization, hemagglutination inhibition (HI), complement fixation (CF), and ELISA (50, 51, 53, 54, 56). Most patients are seropositive by IgM ELISA at the onset of illness. The neutralization assay is positive in most patients by the end of the first week of illness, and neutralizing antibody appears to persist for life. HI antibodies appear with neutralizing antibodies but are not as persistent, and CF antibodies appear only after two to three weeks of illness and are absent within a year (51).

Prevention

Infection with California encephalitis serogroup viruses may be prevented by use of insect repellants and by mosquito control through insecticide spraying and through control of breeding sites. Some communities have attempted removal of discarded tires and other artificial breeding sites, but control of natural breeding sites such as tree holes in woodlands is probably impractical.

Isolation of infected persons is not necessary. There is no known role for passive immunoprophylaxis or antiviral chemoprophylaxis. No vaccine is available, although immunization of mice with a vaccinia recombinant that expresses La Crosse virus glycoproteins protects mice from lethal challenge (57).

Treatment

Treatment of California encephalitis is supportive.

HANTAVIRUSES

Epidemiology

Hantavirus Cardiopulmonary Syndrome (HCPS)

Hantavirus cardiopulmonary syndrome (HCPS) was first recognized in 1993 following a cluster of unexplained deaths in young adults in the rural, Four Corners area of northwestern New Mexico and northeastern Arizona (58–60). Sin Nombre virus, a previously unrecognized hantavirus associated with the deer mouse, *Peromyscus maniculatus*, was found to be responsible for most HCPS cases. Specific serologic tests and tests for viral RNA by reverse transcriptase-polymerase chain reaction (RT-PCR) were quickly developed (35, 59, 61–64). Notably, serologic surveys retrospectively documented cases from as early as 1959 (15, 65, 66). To date, cases have been reported in 31 states in the continental United States, of which over 95% have occurred in states west of the Mississippi (67). Through 6 January 2016, 690 cases of HCPS have been reported in the United States; 109 cases have been reported in Canada through December 2014. There is a male predominance, and the mean age is 38

(range 5 to 84) in the United States and 40 in Canada. Among US cases, 78% have been white, 18% Native American, 19% Hispanic, and 1% African American. The overall case fatality rate among reported cases is 36% in the United States. Cases occur year-round, but most occur in the spring and summer.

Although Sin Nombre virus (SNV) remains by far the most important Bunyavirus pathogen in the United States and Canada, a limited number of HCPS cases in the United States have occurred outside the range of the deer mouse (Figure 2) and are caused by distinct species. These include Black Creek Canal virus (BCCV) from the cotton rat, *Sigmodon hispidus*, in Florida (68, 69), New York virus (NYV), from *P. leucopus* (white-footed mouse) in the Northeastern United States (70–72), and Bayou virus (BAYV) from rice rats (*Oryzomys palustris*) in Louisiana and Texas (73, 74). NYV is closely associated with SNV, whereas BCCV and BAYV are more closely associated with ANDV.

HCPS has also now been reported in Argentina, Canada, Bolivia, Brazil, Chile, Panama, Paraguay, and Uruguay. ANDV, which is carried by the long-tailed pygmy rice rat, *Oligoryzomys longicaudatus*, causes severe HCPS along the southern Andes Mountains in central and southern Argentina and Chile. Andes virus is of particular concern because of strong evidence for person-to-person transmission (41, 75–77). Laguna Negra virus (host *Calomys laucha*) is a recognized pathogen in Paraguay and Bolivia, and Juquitiba virus is a pathogen in Brazil. In Panama, Choclo virus was identified in patients and in the host, *Oligoryzomys fulvenscens*, in an outbreak in 2000 (78).

In Chile, where 759 ANDV cases were reported through February 2013, a mean of 55 cases are reported annually, and the case fatality rate is 32%. The mean age (32 years) is lower than in the United States, in part because ANDV causes disease in young children whereas SNV does not. Through the end of 2006, 841 cases were in Argentina (79) and similar numbers of cases have also been reported in Brazil. As such, both the number of cases and deaths from HCPS in South America exceed the number of cases and deaths in the United States and Canada by a factor of at least three.

In addition to the viruses implicated in HCPS, hantaviruses that have been identified in New World rodents include Rio Mamore virus amplified by RT-PCR from *Oligoryzomys microtus* collected in Bolivia in 1985, Calabazo virus (host *Zygodontomys brivicauda*) in Panama (78); Prospect Hill virus in the meadow vole, *Microtus pennsylvanicus*; Isla Vista virus in the California meadow mouse, *M. californicus*; Bloodland Lake virus in the prairie vole, *M. ochragaster*; El Moro Canyon virus in the western harvest mouse, *Reithrodontomys megalotis*; and Rio Segundo virus from *R. mexicanus* (80–84).

Sero-epidemiology

Current evidence suggests that subclinical infections with SNV and ANDV are uncommon. Although a small number of individuals without a history of HCPS, including mammalogists and persons living in rural areas of the Four Corners region of New Mexico and Arizona, have been found to have hantavirus antibodies, it is not clear whether these individuals have been infected with SNV. Surveys of hundreds of individuals presenting to healthcare facilities in the Four Corners region and equal numbers of national park workers and others presumed to have high-risk occupations have failed to identify seropositive persons (85, 86). Seroprevalence rates of 3 to 30% have been reported in some areas in Central and South America. Rates as high as 12% have been reported in some high-risk areas in Chile when testing was done by the CDC EIA using standard cutoff values, whereas seroprevalence rates were much lower (3%) and there was intertest agreement when samples were tested by the strip immunoblot assay and by neutralizing antibody testing and a higher cutoff was used for the EIA (P. Vial, personal communication). Seroprevalence rates exceeding 30% have been reported in Panama, where Choclo virus causes a mild form of HCPS with a low case fatality rate and

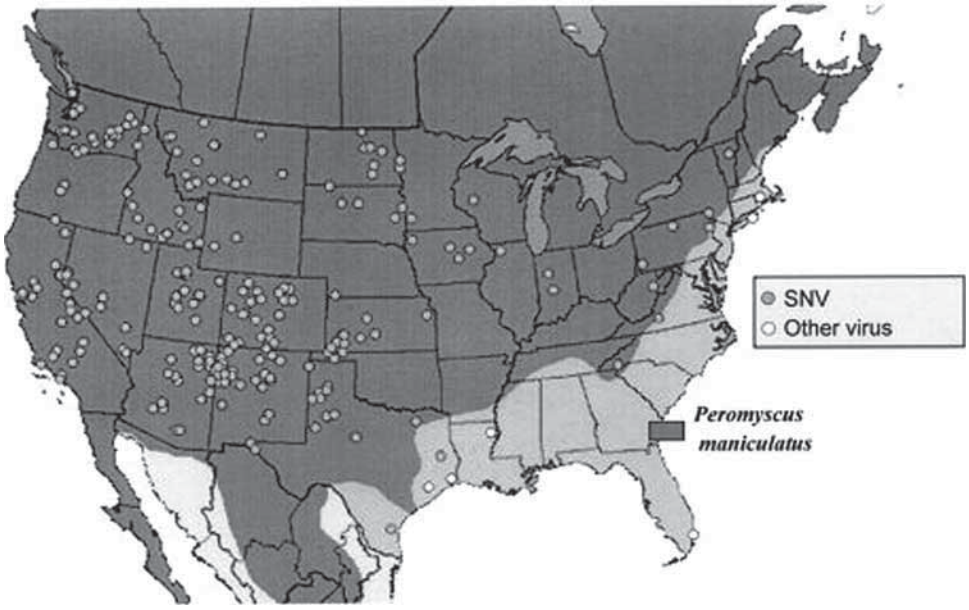


FIGURE 2 Distribution of *Peromyscus maniculatus* and location of HPS cases as of 9 May 2006. Total cases (N = 438 in 30 states) (from Centers for Disease Control and Prevention).

where a high proportion of seropositive individuals have no history of HCPS (87). Although subclinical infections with SNV and ANDV may be rare, mild, symptomatic infections are probably underreported for all hantaviruses that cause HCPS. A number of persons have been identified in New Mexico, Chile, and elsewhere that have not been hospitalized or only hospitalized for a few days.

Risk Factors

Most persons with HCPS lived in rural areas or had occupations or recent activities that involved exposure to rural areas (85, 86, 88). In case-controlled studies, the risk factors for HCPS included seeing increased numbers of small rodents in the home, cleaning home food storage areas, cleaning feed storage areas (barns), and cleaning animal sheds (86, 88, 89). Of note, risk factors almost always include indoor exposure (89). A 2012 outbreak in Yosemite National Park (10 cases with 3 deaths) was linked to deer mice nesting in the insulation of tent cabins (90).

Risk factors for person-to-person transmission of ANDV infection include close contact with the index case before or shortly after the onset of symptoms rather than following development of the cardiopulmonary phase when patients typically present for hospital admission (75, 91, 92). In a prospective study of 476 household contacts of index cases with HCPS in Chile, the risk of HCPS was 17.6% in sex partners versus 1.2% in other household contacts ($P < 0.001$), and 32.6% of 96 HCPS cases occurred in household clusters with two or more cases in the household (75). Despite conclusive evidence for person-to-person transmission with ANDV among close household contacts, nosocomial transmission is exceedingly rare. While nosocomial transmission was suspected in the 1996 outbreak in Argentina (77, 93) clear evidence of nosocomial transmission of ANDV has been documented in a single case cluster in Chile (41).

Hemorrhagic Fever with Renal Syndrome (HFRS)

The first recognized hantavirus associated with HFRS was Hantaan virus (HTN) transmitted by *Apodemus agrarius* (striped field mouse) and distributed throughout the Far East, particularly in China and Korea, eastern Russia, and the Balkans (Table 2) (6, 36, 48, 94–100). Another severe form of HFRS associated with Dobrava (DOB) virus from *Apodemus flavicollis* (yellow necked field mouse) occurs in the Balkans (101, 102). Saaremaa virus, carried by *A. agrarius* (striped field mouse), causes mild HFRS and occurs in eastern and central Europe. Another mild form of HFRS associated with Seoul virus (SEO) and *Rattus norvegicus* (brown rat) occurs worldwide (6, 37, 38, 95, 100–105). The mildest form of HFRS, also denoted nephropathia epidemica, is caused by Puumala virus (PUUV), which is carried by *Myodes glareolus* (bank vole); PUUV occurs throughout Scandinavia, western Russia, and central Europe (39, 95, 100, 106–108).

Although infection with the hantaviruses causing HFRS occurs in all age groups, infection and disease peak in adults 15 to 40 years of age; like HCPS, HFRS is uncommon in children (109–111). There may be 150,000 cases of HFRS per year with the majority in Asia. In an agricultural district in China, the ratio of subclinical to clinical hantavirus infections was 5.4:1 based on cross-sectional data versus 14:1 based on prospective data, and clinical infections were more likely to be caused by Hantaan than by Seoul virus. Among rodents trapped in the area, most Hantaan infections were found in *A. agrarius*, whereas most *Seoul* infections were found in *Rattus norvegicus* trapped in homes (110).

Risk Factors

Human infections with Hantaan virus occur primarily in adults in rural areas. Some studies report a male preponderance of Hantaan virus infection in men (2:1), but rates among men and women may be equal in areas where agricultural work is performed near the home or where crops are stored near homes during winter months (109–111). In the latter case, the field mice may be likely to invade homes and food storage areas. Specific occupational groups, including farmers, forest workers, and field soldiers, have been shown to be at higher risk, as have activities such as harvesting and sleeping in straw huts (111).

Seoul infection appeared to account for approximately 20% of infections and < 5% of cases of HFRS in rural China, but Seoul virus appears to cause most urban outbreaks due to the distribution of the reservoir host, the brown rat. Large outbreaks have been described in Osaka, Seoul, and seaports in China, but limited outbreaks have been described in seaports throughout the world.

Puumala virus–associated HFRS (also called nephropathia epidemica) primarily occurs among farmers and others living in rural areas (107, 112–114). In Scandinavian countries, where more than a thousand cases occur annually, most cases occur in the fall and early winter when bank voles infest homes and peridomestic buildings (115). In Europe, cases are more frequent in relation to rodent population peaks every 3 to 4 years (115, 116). Seroprevalence in endemic areas of Sweden is approximately 10% (114). The male to female ratio ranges from almost 2:1 to almost 5:1. The peak ages for Puumala infection and disease are similar to those for Seoul and Hantaan infections. Fewer than 5% of persons with PUUV infection are <15 years of age (117). There are approximately 100 hospitalizations a year in Sweden with serosurveys suggesting 10 to 20 infections for every hospitalized case (114, 118).

Transmission

As indicated above, humans acquire hantavirus infections by aerosol transmission from inhalation of infected rodent excreta, including urine, droppings, or saliva (100, 117). There is no evidence of human-to-human transmission of the viruses that cause HFRS, including nosocomial transmission in hospitals. Seroconversion and clinical infection have occurred among animal handlers and persons visiting the laboratories housing small rodents, and, rarely, following bites and dissection of infected animals and in persons working with hantaviruses in cell culture (117, 119). Transmission through blood products has been reported (120).

Pathogenesis in Humans

The incubation period between rodent contact and onset of symptoms is difficult to determine, since the period of potential exposure may be prolonged. A small study of persons with PUUV infection suggested that the median was about four weeks, with a range of one to eight weeks (121). For the more severe forms of HFRS, the average incubation period has been estimated to be 21 days, with a range of four days to six weeks (111, 122, 123). For HCPS the median incubation period was reported as 18 days (range of 10 to 34 days) in a small series of 11 patients with ANDV-associated HCPS in Chile (124). In another report, the potential period of exposure was limited to 48 hours or less, and the median incubation period was 30.5 days (range 20 to 49 days) among

10 persons who acquired SNV infection at the Yosemite National Park in California in 2012 (90).

Viral Replication Patterns

The role of viremia in the pathogenesis of HFRS and HCPS is not known. However, in household contacts ANDV RNA could be routinely detected in peripheral blood for up to 15 days before the onset of symptoms or development of antihantavirus antibodies (75), and PUUV RNA has been detected in a serum sample obtained at the time of an incidental urinary tract infection three weeks prior to symptom onset in a case of severe HFRS (125). High levels of viral RNA can be detected by quantitative RT-PCR in patients with SNV-associated HCPS at the time of hospital admission, and there is a significant association between high viral load at admission and severe disease (126).

Virus isolation from humans with HFRS is difficult (73, 127, 128); there has been only one successful isolation of ANDV from the serum of a 10-year-old, seronegative Chilean boy two days before he developed fever and six days before he died from HCPS (129). Furthermore, in a recent prospective study of household contacts of index patients with HCPS in Chile, ANDV RNA was detected by RT PCR in peripheral blood cells 5 to 15 days before the onset of symptoms or detection of antihantavirus antibodies in four household contacts who subsequently developed HCPS (75).

In persons who die from HFRS and HCPS, hantaviral antigens are widely distributed in organs as demonstrated by immunohistochemical stains (130, 131). In HCPS these include lung, kidney, heart, spleen, pancreas, lymph nodes, adipose tissue, skeletal muscle, intestine, adrenal gland, and brain; in contrast, only rare sinusoidal lining cells are stained in the liver (131). Viral antigens are detected primarily within capillary endothelial cells but are rarely detected in large veins or arteries. In HCPS, the most intense staining is found in pulmonary capillary endothelial cells. In the kidney, immunostaining is found in interstitial capillaries in the medulla and cortex and in glomerular endothelial cells. In contrast, in persons dying of HFRS, immunohistochemical staining is most intense in the kidney. Immunohistochemical staining in tubular epithelial cells is prominent in HFRS but rare in HCPS.

Electron microscopic (EM) studies of HCPS autopsy tissue demonstrate infrequent hantavirus inclusions in pulmonary capillary endothelial cells, and, more rarely, virions in both pulmonary capillary endothelial cells and interstitial macrophages (131, 132). Hantaviruses have been infrequently recovered from humans, so the duration of viremia and viral excretion has not been clearly determined. However, SNV RNA has been uniformly detected in peripheral blood mononuclear cells (PBMC) by RT-PCR from persons hospitalized with HCPS, but only intermittently in specimens collected 16 to 23 days after onset of symptoms and not in later specimens (133). In contrast, SNV RNA was detected by RT-PCR in only one of three bronchoalveolar lavage samples in patients in whom SNV RNA was easily detected in PBMC.

Histopathology

Autopsies of HFRS cases show capillary engorgement and focal hemorrhages widely distributed in the kidneys and hemorrhagic necrosis of the renal medulla (134). Retroperitoneal edema is present in persons who die of shock early in the course of illness. Widespread hemorrhages with or without hemorrhagic necrosis occur in the subepicardium and epicardium of the right atrium, anterior pituitary, meninges and subarachnoid space, pancreas, and skin. Hemorrhage and necrosis are most marked in the right atrium in patients who have died early in the shock phase, whereas hemorrhagic necrosis of the anterior pituitary has been most prominent in patients who survived the shock phase and died during the oliguric or diuretic phases (see below). Although pulmonary edema has been described in HFRS, most cases of fatal pulmonary edema from the Korean War occurred during the oliguric and diuretic phases rather than the shock phase (134–137). Pulmonary interstitial infiltrates also appeared much less prominent in HFRS than in HCPS (131, 132, 134–136). When using modern radiologic techniques, high-resolution computed tomography showed thoracic effusions or pulmonary edema in almost half of HFRS patients infected with PUUV (138). In both HFRS and HCPS, there were infiltrates of large, atypical mononuclear cells in the spleen, lymph nodes, and in the hepatic portal triads. Renal biopsies of patients with NE have shown interstitial edema and hemorrhage, tubular degenerative changes, and glomerular inflammatory infiltrates, and changes consistent with acute tubular necrosis may persist for several months (136).

In a series of 13 autopsies of patients with HCPS performed at the University of New Mexico, all had large, bilateral pleural effusions, and large, rubbery, edematous lungs with significantly increased weight (132). Splenomegaly was found in 5 of 12, and gastric mucosal hemorrhage was found in 3 of 13. Histologically, the lungs showed intra-alveolar and septal edema, interstitial infiltrates with mononuclear cells (primarily T lymphocytes with a CD4/CD8 ratio about 2:1 and macrophage/monocytic cells), and sparse to moderate hyaline membranes (132). Large immunoblasts, primarily activated CD8+ cells, were found in pleural fluid and peripheral blood (Figure 3) (132, 139). In contrast to findings in patients with adult respiratory distress syndrome, the type I alveolar lining cells were intact and type II lining cells were not activated; hyaline membranes were composed primarily of fibrin, and neutrophils and cellular debris were uncommon (Figure 4). In contrast to the findings in HFRS, gross and histologic findings in patients with HCPS were normal in the brain, pituitary, heart, kidneys, adrenals,

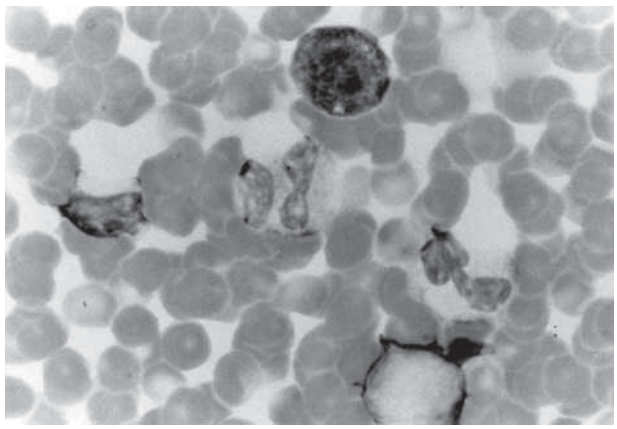


FIGURE 3 Peripheral blood smear from a patient with the cardiopulmonary stage of hantavirus cardiopulmonary syndrome. Note the immunoblast with basophilic cytoplasm, prominent nucleolus, and high nuclear to cytoplasmic ratio. Also note immature neutrophils. Courtesy of R. Feddersen.

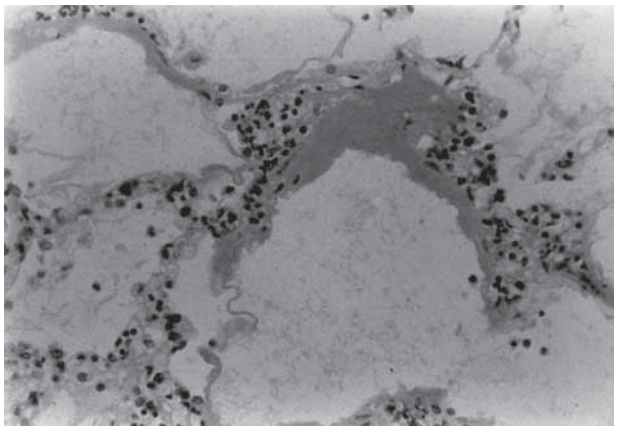


FIGURE 4 Pulmonary histology in a patient who died from hantavirus cardiopulmonary syndrome. Note the intra-alveolar and septal edema, interstitial infiltrates with mononuclear cells, and sparse hyaline membrane. In contrast to patients with ARDS, the hyaline membranes are largely devoid of inflammatory cells and cellular debris. Courtesy of Dr. K Nolte.

pancreas, and skin, and no patient had retroperitoneal edema (132). Of note, pathologic evidence of myocarditis has been reported in patients who died from HCPS (140).

Immune Responses

Both HCPS and HFRS cause capillary leak syndromes. The differences between the two syndromes include the primary target organs (the lung in HCPS and the kidney in HFRS) and lack of widespread hemorrhage and hemorrhagic necrosis in HCPS despite the presence of thrombocytopenia in both syndromes. Although capillary endothelial cells are infected with hantaviruses in both syndromes and the degree of hantavirus infection is greatest in the capillary endothelial cells in primary target organ, EM studies in both syndromes have failed to show any evidence of capillary endothelial cell necrosis or cytotoxicity. Thus, it has been postulated that virus-infected endothelial cells may be injured by T cells, cytokines, or other immune-mediated factors creating gaps between endothelial cells (139). Recently, it was shown that hantavirus infected endothelial cells actually were protected from injury by cytotoxic cells and apoptosis was inhibited (133, 141). In both syndromes, antihantavirus antibodies are invariably present at the onset of clinical involvement of the primary target organ. In HCPS, elevated plasma levels of IL-2 and its receptors, gamma-interferon, IL-6, and soluble receptors for TNF suggest marked cytokine activation, and the same is suggested by increased levels of soluble IL-2 receptor, TNF, kallikrein-kinins, and by gamma-interferon expression in HFRS. Circulating immune complexes, activation of the complement pathway, and deposition of immunoglobulins and complement in both the glomerular basement membrane and tubules are present in HFRS but not HCPS.

Early development of high neutralizing antibody titers to Sin Nombre virus is associated with mild HCPS. Patients with severe or fatal acute SNV and PUUV infection have significantly lower neutralizing antibody titers on the day of hospital admission compared with patients with mild disease (142, 143).

β_3 integrins mediate cellular entry of pathogenic hantaviruses (Hantaan, Seoul, Puumala, Sin Nombre, and New York viruses) but do not mediate the entry of Prospect Hill virus, a virus not associated with human disease, in human umbilical vein endothelial cells and in Vero E6 cells (24, 144). β_3 integrins are present on platelets, endothelial cells, and macrophages and are also known to regulate vascular permeability and platelet function.

Humoral and cell-mediated immune responses appear to persist for decades, and recurrent episodes of HFRS or HCPS have not been reported. Thus, it is presumed that infection leads to lifelong protection from repeat infection.

Clinical Manifestations

Hantavirus Cardiopulmonary Syndrome (HCPS)

The illness typically begins with a febrile prodromal phase characterized by fever and myalgia; the latter is often prominent (Figure 5) (145). Headache, backache, abdominal pain, diarrhea, nausea, and vomiting may also be present, particularly after the first 24 to 48 hours. As with HFRS, fever and abdominal pain may dominate the clinical presentation suggesting acute appendicitis or another cause of an acute surgical abdomen (Table 5). The febrile phase, which usually lasts 3 to 4 days but may last 10 to 12 days, is followed by the sudden onset of noncardiogenic pulmonary edema and, in some cases, by shock (58–60, 146).

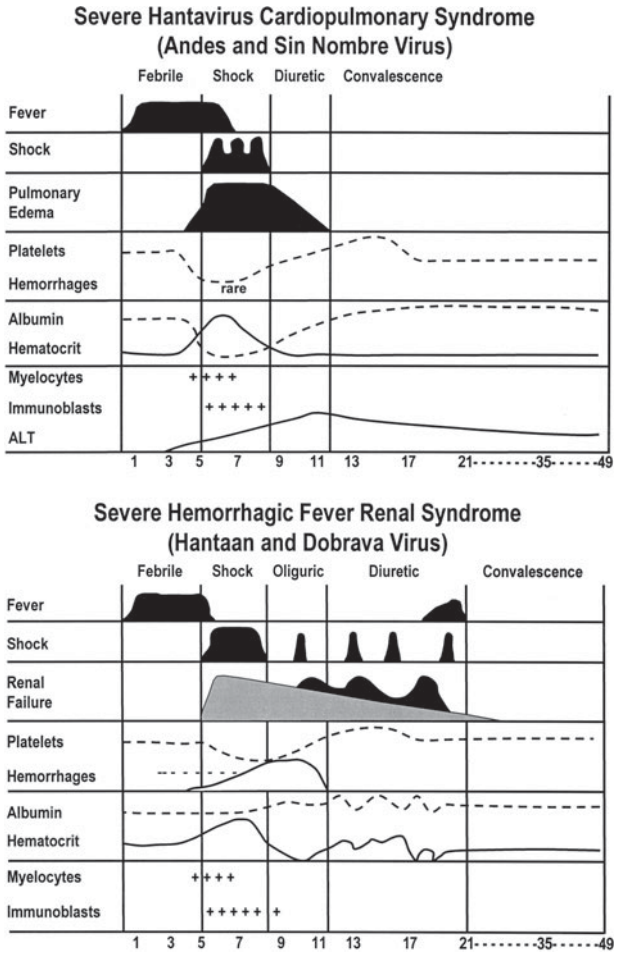


FIGURE 5 Clinical course and typical laboratory findings in severe hantavirus cardiopulmonary syndrome and severe hemorrhagic fever with renal syndrome. Reprinted from reference (145) with permission of the publisher.

TABLE 5 Clinical and laboratory features of Hantaan-associated hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS)

Abnormality	HFRS	HCPS
Prodrome with fever and myalgias	Present	Present
Facial flushing	Common early	Absent
Petechiae	Common late in prodrome	Absent in Sin Nombre virus, often present with Andes virus
Conjunctival injection	Common early	Absent
Pulmonary edema	Uncommon except in oliguria	Present during cardiopulmonary stage
Proteinuria	Present with onset late in prodrome	Uncommon
Hemorrhage	GI, CNS and right atrial hemorrhage seen in severe cases/deaths	Rare with Sin Nombre virus; clinical bleeding (from venipuncture sites, pulmonary hemorrhage) more common with Andes virus
Renal failure/azotemia	Common	Uncommon/mild
Hemoconcentration	Uncommon except with 9BP/shock	Present in 50% of patients
Thrombocytopenia	Present early	Present early
Nonproductive cough/ shortness of breath	Uncommon	Present at onset of cardiopulmonary stage
Shock	Uncommon	Common during cardiopulmonary stage
Hypotension	Common	Very common

The cardiopulmonary or shock phase of HCPS is heralded by the abrupt onset of cough and shortness of breath, which may be accompanied by dizziness. Pulmonary edema develops rapidly, usually over 12 hours or less. All hospitalized cases have become hypoxic during this stage, and nearly all have required supplemental oxygen. Approximately 75% of cases require mechanical ventilation, and mortality is approximately 50% in those who require intubation. Monitoring with a flow-directed pulmonary artery catheter typically shows normal pulmonary wedge pressure (PWP), decreased cardiac index (CI), and elevated systemic vascular resistance (SVR) (Table 6). These parameters are helpful in clinically differentiating HCPS from septic shock. In the latter, the CI is typically elevated and the SVR is depressed.

Among hospitalized patients, death is usually preceded by the abrupt onset of a profound lactic acidosis, cardiogenic shock, and by pulseless electrical activity despite the ability to maintain adequate oxygenation. Death may occur within the hours of the first pulmonary symptoms, with death being uncommon after approximately 48 hours of intubation. A lactate ≥4 mmol/l, marked hemoconcentration, cardiac index ≤2.2, and persistent hypotension are indicators of a poor prognosis. Among hospitalized patients, death almost always results from shock with very low cardiac output and from cardiac arrhythmias. Death rarely occurs from respiratory failure provided that mechanical ventilation is available. As such, this chapter and many recent publications use the term hantavirus cardiopulmonary syndrome (HCPS) rather than hantavirus pulmonary syndrome (HPS) to call attention to the important role of shock and arrhythmia in serious and fatal HCPS.

TABLE 6 Hemodynamic summary at clinical nadir of 8 HCPS patients with shock

	HCPS (mean ± SEM)	Normal (range)
Cardiac index (l/min/m^2)	2.1 ± 0.33	2.5–4.2
Pulmonary artery wedge pressure (mm Hg)	15.6 ± 2.6	8–10
Systemic vascular resistance index (dyne.sec.cm^{-5}/m^2)	2,114 ± 258	1,700–2,500
Pulmonary artery mean (mm Hg)	29.7 ± 3.5	8–20
Stroke volume index (ml/beat/m^2)	17.9 ± 3.5	33–47

Clinical nadir defined by lowest value of stroke volume index. All patients were receiving vasoactive or inotropic drugs at the time of measurement and had received volume resuscitation. Reprinted from reference (146) with permission of the publisher.

After two to four days, recovery is heralded by the onset of the diuretic phase. Clinical improvement is often rapid, allowing many patients to be extubated within 12 to 24 hours after the onset of diuresis. Supplemental oxygen may be required for several days following extubation. The diuretic phase is typically followed by a convalescent phase that may last up to several months and is characterized by weakness and fatigue, but limited long-term follow-up studies suggest that patients recover fully without residual abnormalities.

Chest radiographs are usually normal during the prodromal, febrile phase and may be normal at the onset of pulmonary symptoms, but radiographic abnormalities invariably appear shortly after the onset of pulmonary symptoms (147). Early findings include radiological signs of bilateral interstitial edema, including Kerley B lines, hilar indistinctness, or peribronchial cuffing. Within 48 hours (often within 2 or 3 hours), radiographic signs of bilateral air space disease develop in a majority of patients, including all who require mechanical ventilation or die (Figure 6).

The platelet count begins to drop shortly after the onset of fever (Figure 5) (145). Isolated thrombocytopenia has been present in samples obtained 24 to 48 hours before the onset of the shock phase. In the shock phase, characteristic hematological abnormalities include thrombocytopenia, increased white blood cell counts with immature granulocytes, ≥10% immunoblasts, and elevation of LDH. Patients with severe disease also exhibit hemoconcentration, hypoalbuminemia, and lactic acidosis. The immunoblasts are characterized by basophilic cytoplasm (occasionally with

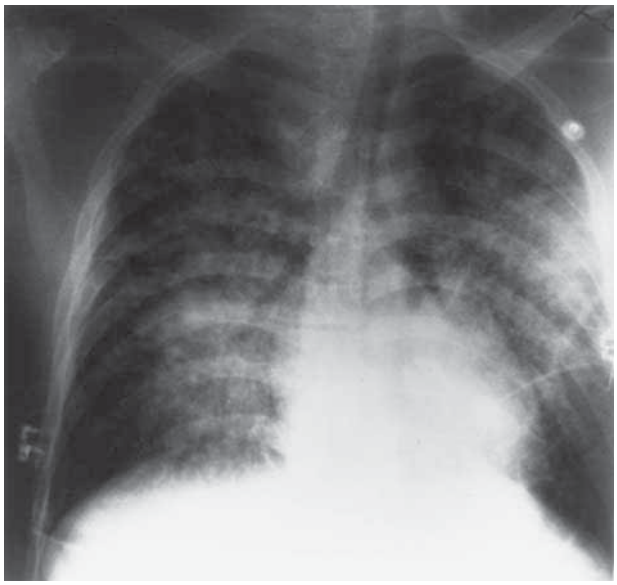


FIGURE 6 Bilateral pulmonary edema in a patient with the cardiopulmonary stage of HCPS. Courtesy of L Ketai.

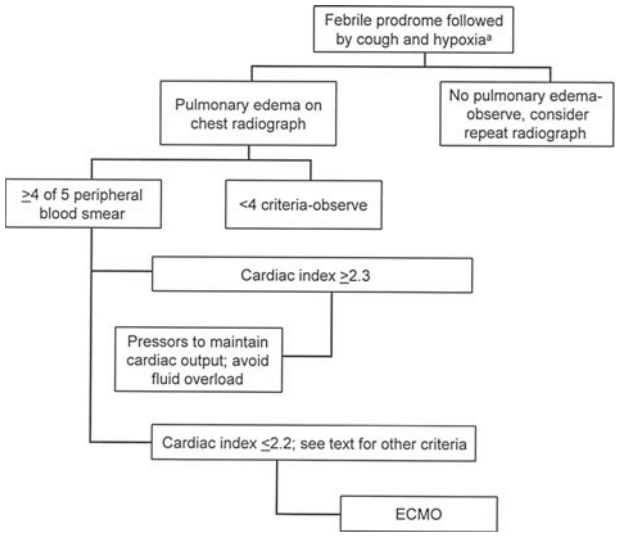


FIGURE 7 Flow diagram for the management of patients suspected of having hantavirus cardiopulmonary syndrome in the cardiopulmonary stage pending IgG and IgM antihantavirus antibody results. Reprinted from reference (148) with permission of the publisher.

coarse granules), prominent nucleoli, and a high nuclear-to-cytoplasmic ratio (Figure 3). Elevations in aspartate aminotransferase typically occur after the onset of the cardiopulmonary/shock phase and peak early in the convalescent phase (Figure 5).

Diagnosis

The diagnosis should be considered in persons who present with fever and moderate to severe myalgia, particularly if the person has had recent rural exposure or a recent high-risk exposure such as cleaning food or feed storage areas, catching rodents, agricultural work, or increased numbers of rodents in the home. Whenever practical, a complete blood count, including a platelet count, should be obtained. In the setting described above, the presence of thrombocytopenia should trigger both serologic testing for hantavirus and consideration of referral to a center with critical care capabilities.

Clinical decisions regarding patient care including institution of ECMO must often be made before the results of serologic testing are available (Figure 7) (148). Fortunately, an initial clinical diagnosis of HCPS can be made with a high degree of confidence in patients who are in the cardiopulmonary phase based on the clinical presentation and a review of the peripheral blood smear. In a recent blinded analysis of patients with a febrile prodrome followed by pulmonary edema, the presence of four or more of the following findings had a sensitivity of 96% and specificity of 99% for diagnosis of HCPS (149): thrombocytopenia, myelocytosis, lack of significant toxic granulation in neutrophils, hemoconcentration, and more than 10% lymphocytes with immunoblastic morphologic features. Thus, pending results of serologic testing, a probable diagnosis can be established for patients in the shock or cardiopulmonary phase of HCPS through examination of the peripheral smear and by measurement of the CI, PWP, and SVR with a flow-directed pulmonary artery catheter or approximated by noninvasive monitoring (146, 148–150).

Differential Diagnosis

The differential diagnosis is broad, but should include pneumonia, sepsis, endocarditis, and septic shock; antibiotic coverage should be provided to cover the most likely clinical syndromes. At the University of New Mexico, antibiotic coverage includes pathogens such as *N. meningitidis*, *S. pneumonia*, *Y. pestis*, and *F. tularensis* unless a specific pathogen or syndrome is suspected. Although SNV has not been transmitted nosocomially, isolation should be considered until the diagnosis is established or until infection with agents such as *N. meningitidis* or *Y. pestis* has been ruled out. Rare nosocomial transmission of ANDV has been reported, so contact and droplet isolation should be utilized for routine care, and N95 respirator masks should be used during procedures such as suctioning and intubation (41).

Unfortunately, it is very difficult to differentiate patients who are in the febrile, prodromal phase of HCPS from patients with other febrile illnesses. The differential diagnosis of fever and thrombocytopenia is broad. For patients with rural exposure in North America, the differential diagnosis varies by region and the type of exposure but could include plague, tularemia, leptospirosis, ehrlichiosis, Colorado tick fever, relapsing fever, or spotless Rocky Mountain spotted fever.

Hemorrhagic Fever with Renal Syndrome (HFRS)

The clinical severity of HFRS varies according to the virus type. Hantaan-associated HFRS, also called Korean and epidemic hemorrhagic fever, has been divided into five stages: febrile, hypotensive, oliguric, diuretic, and convalescent (Figure 5). Not all patients with Hantaan-associated HFRS have all five stages, and the stages appear somewhat less useful in understanding the clinical course of HFRS caused by the other viruses (136, 151–153).

Hantaan-associated HFRS

The illness of Hantaan-associated HFRS begins with the onset of fever, which is universal and lasts from three to seven days (136, 151–153). Symptoms like malaise, myalgias, and headache are common, as are flushing of the face and neck in conjunction with periorbital edema and conjunctival injection or hemorrhage (Table 5). After several days, the symptoms may worsen and be accompanied by

nausea, vomiting, abdominal pain, and lower back pain. Petechiae may be found, particularly on the head, neck, and trunk and palate. Laboratory findings include a normal or slightly elevated WBC and decreasing platelet count. Proteinuria is present, gradually increases during this phase, and persists through the oliguric phase.

Coincident with or shortly before resolution of the fever, some patients develop hypotension and shock. Mild degrees of hypotension may develop in up to 50% of patients, whereas shock develops in 10 to 15%. In patients with shock, the WBC is markedly elevated with a left shift and immature forms; there is marked thrombocytopenia and hemoconcentration. Patients at this stage may exhibit more widespread petechiae and bleeding, and disseminated intravascular coagulation is usually present (136, 151, 152). In the remainder, the hypotensive phase resolves after a period of several hours to two or three days.

During the second half of the first week of the illness, oliguric renal failure develops in up to 60 to 70% of patients. If present initially, hypotension and elevations in the hematocrit resolve during the oliguric phase, but hypervolemia with pulmonary edema, neurologic complications, and increased bleeding may occur. Dialysis may be required, particularly in patients with renal failure and pulmonary edema. In a recent series, oliguric renal failure was present for a mean of 8 days (range 3 to 17 days) (136). Deaths may occur secondary to pulmonary edema, electrolyte disturbances, shock, and CNS and other hemorrhages.

The diuretic phase begins about the middle of the second week of illness. Patients who have had mild disease generally recover quickly during this phase, but more severely ill patients are at risk of death from intravascular volume depletion, electrolyte disturbances, and secondary infection. This stage may be brief in patients with mild disease or may gradually resolve over several weeks in those with more severe disease; the mean duration is about 6 days (range 2 to 12) (136). The diuretic phase may be followed by a prolonged convalescent phase.

The risk of death from Hantaan-associated HFRS during the Korean War was about 15% but more recently has been 5 to 7%. A recent publication from China reported an overall case-fatality rate of 1.2% (154). Among survivors during the Korean war, four of five who developed pulmonary edema during the oliguric phase required hemodialysis, suggesting that the risk of death during the oliguric and diuretic phases may have been reduced by critical care treatment and hemodialysis (136). Enhanced clinical care has improved the outcome of HFRS, although in many HFRS endemic countries it is difficult to determine the causative hantavirus and estimated case-fatality rates may be confounded by including both Seoul and Hantaan virus infections.

HFRS from Seoul, Saarema, and Dobrava Viruses

Seoul-associated HFRS is less severe and has fewer distinct phases with overall mortality estimated at less than 1%. Hemorrhagic manifestations appear less common, whereas abdominal pain, hepatomegaly, and hepatic dysfunction may be more common. Serologic evidence of hantavirus infection was found in 6.5% of patients with end-stage renal disease due to hypertension in Baltimore (155, 156). These data suggest that a hantavirus may cause hypertension and end-stage renal disease but need to be confirmed by other groups and with prospective studies. Although the serologic data suggest that the antibodies react most strongly to Seoul virus, hantaviral RNA should be examined to determine whether the syndrome is caused by Seoul or a closely related virus. Saarema virus is also reported to cause a mild form of HFRS. Dobrava virus, in contrast, causes a severe form of HFRS with mortality of 9 to 12%, hypotension in almost 50%, and oliguric renal failure requiring dialysis in 30 to 47% (115). Hemorrhage or hemorrhagic complications are reported in 9 to 26%, and shock is reported in 21 to 18% (115).

HFRS from Puumala Virus, Nephropathia Epidemica

Mild HFRS caused by PUUV infection, also denoted nephropathia epidemica, has a mortality estimated to be less than 0.5% (115, 118), but may be considerably higher for elderly patients (157). The syndrome begins with a fever that typically lasts between two and nine days. Within one to two days of the onset of fever, most patients also develop headache, malaise, and backache. Abdominal pain has been reported in most patients, and, as with other hantavirus infections, this presentation may mistakenly suggest an acute surgical abdomen. Hypotension occurs but overt shock is rare. Disseminated intravascular coagulation and minor hemorrhagic manifestations such as macroscopic hematuria and epistaxis were reported in around one-third of patients (121, 158). Proteinuria (almost 100%), elevated creatinine (in almost 100%) with peaks of 2 to 10 mg/dl, and oliguria ($\backsim$50% of cases) typically begin at about the third or fourth day of illness and last only a few days (115). This phase is followed by a diuretic phase that lasts for a week to ten days. Rarely patients with NE have also been reported to have encephalitis, Guillain-Barré syndrome, and disseminated encephalomyelitis as well as acute perimyocarditis (115, 159). CSF examination is usually normal but may show slight elevations in protein and white blood cells. Blurred vision has been reported in about 30% of cases (160). This phenomenon is due to myopia that may be caused by ciliary body and lens edema (161). The mean duration of hospitalization has ranged from 8 to 22 days, and the median time of missed work ranges from 36 to 48 days (121).

Laboratory Diagnosis

Direct virus isolation from humans with HFRS and HCPS is difficult (129). In addition, virus isolation requires BSL-3 facilities, and animal models for HCPS require BSL-4 animal facilities (40).

Hantavirus Cardiopulmonary Syndrome

As in the case of HFRS, specific IgG and IgM antibodies have been almost uniformly present in the first serum samples tested (60, 63, 162), including testing of limited numbers of serum samples obtained early in the febrile prodrome. Available serologic tests include ELISA, neutralization, hemagglutination inhibition (HI), Western immunoblot, and strip immunoblot assays.

In the United States, serologic testing to date has used ELISA and Western and strip immunoblot assays. The Centers for Disease Control and Prevention (CDC) has developed an IgG ELISA and an IgM-capture ELISA based on recombinant N proteins of SNV. Commercial ELISA antibody testing is for hantavirus; testing for SNV antibodies is also available through Focus Technology, but the antigens employed in these assays are proprietary. N protein is highly immunogenic and tends to be cross-reactive among hantaviruses. Inclusion of the N protein enhances the sensitivity of an assay, and cross-reactivity may be useful as in the case when one is performing serologic screening for a previously unrecognized hantavirus. However, it is difficult to differentiate between hantavirus species using the N protein.

In the Western immunoblot format, the first 59 amino acid (aa) residues of SNV N protein contain almost all the epitopes of the 428 protein, and the dominant linear epitope of SNV G1 protein is confined to a variable region at residues 58 to 68 (63, 163). The latter elicits little or no cross-reactivity among hantaviruses but is conserved among SNV from different regions.

Quantitative measurement of neutralizing antibody is limited to research laboratories. A plaque reduction assay is available, but this assay takes several weeks to perform and must be performed in a BSL-3 laboratory. More recently, rapid, BSL-2 based neutralization assays have been developed utilizing VSV pseudovirions expressing hantavirus glycoproteins (164).

Viral RNA can be readily detected by RT-PCR in peripheral blood mononuclear cells in persons with acute SNV or ANDV infection. Post mortem, hantavirus infection can be documented with serologic studies or by examination of tissue (particularly lung tissue) by RT-PCR or by immunohistochemical studies using monoclonal antibodies. Sequence analysis is helpful in determining the hantavirus species and has proven useful in molecular epidemiological studies (35, 41, 61, 62, 64, 70, 71, 76, 81, 105, 129).

Hemorrhagic Fever with Renal Syndrome

In eastern Asia, infection with Hantaan and Seoul hantaviruses is usually diagnosed serologically with IFA, ELISA, or bead-agglutination formats, and IgM antibody is determined by IgM-capture ELISA. Similarly, Puumala hantavirus is commonly diagnosed by detection of specific IgG and IgM antibodies by IFA, ELISA, or RIA (112, 113, 115). As in the case of HCPS, IgM antibodies are almost invariably present at or within days of the onset of symptoms. Viral RNA is detectable from blood or serum by RT-PCR in 90% the first three days after onset of disease and in 81% of patients when collected at day 4 to 7 (143) and in less than two-thirds of patients with acute Dobrava virus infection (115, 165).

Prevention

General guidelines for prevention of infection with hantaviruses include efforts to seal sites of potential rodent entry into homes and peridomestic buildings (particularly storage sheds), careful attention to prevent rodent access to food, and by discouraging rodent nesting by eliminating potential nesting sites around the home and workplace. Detailed guidelines for cleaning up rodent-infested areas and for working with potentially infected rodents have been published (166, 167). There is no known role for passive immunoprophylaxis or antiviral chemoprophylaxis, but animal models using HTNV suggest that antiviral chemoprophylaxis with intravenous ribavirin would probably offer some protection from clinical infection. In addition, passive administration of anti-ANDV neutralizing antibody four or five days after ANDV infection was protective in the Syrian hamster model (168).

Candidate recombinant Hantaan vaccines, including a vaccinia recombinant vaccine and a baculovirus-expressed subunit vaccine, have been developed at USAMRIID (169, 170, 174, 175). Vaccines with both G1 and G2 of Hantaan virus can elicit neutralizing antibody responses and provide partial protection from challenge in hamsters, and administration of baculovirus-expressed N protein also prevented virus expression in hamsters. However, these vaccines have been dropped from development, since the vaccinia recombinant vaccines were poorly immunogenic in humans, and the baculovirus products did not produce sufficient quantities of purified antigen (171, 176). Investigational strategies include DNA vaccination for pathogenic hantaviruses, including HTNV, SNV, and ANDV (164, 168).

Formalin-inactivated tissue culture–derived Hantaan and Seoul vaccines have been developed in Asia. A formalin-inactivated, mouse-brain-derived Hantaan vaccine has been licensed and widely used in Korea since 1990 (172, 177). The vaccine is immunogenic and well tolerated, but rigorously performed, controlled efficacy trials have not been reported. In China, formalin-inactivated Hantaan virus vaccines have been prepared in Mongolian gerbil kidney cells (MGKC) or golden hamster kidney cells (GHKC) (173). These vaccines appear immunogenic, and field trials suggest efficacy. However, neither of the vaccines has been tested in randomized, placebo-controlled trials. A bivalent vaccine with inactivated Hantaan and Seoul virus has also been developed in China (173). The bivalent vaccine appears immunogenic, but efficacy trials have not been reported. Three doses are required to obtain protection according Chinese reports. Incompletely vaccinated patients display a milder course of HFRS infection than unvaccinated ones (174).

Treatment

Hantavirus Cardiopulmonary Syndrome

Principles of critical care management for patients with the cardiopulmonary syndrome include oxygen delivery with intubation and ventilatory support as necessary (146). Because of the high potential for rapid clinical deterioration, whenever feasible patients should be transferred to a facility with sophisticated critical care facilities, ideally including the availability of extracorporeal membrane oxygenation (ECMO). Monitoring of cardiac index, pulmonary wedge pressure, and systemic vascular resistance with a flow-directed pulmonary artery catheter should be performed or approximated using noninvasive monitoring if feasible. Inotropic support and vasopressors should be employed rather than fluids whenever possible to reduce the likelihood of worsening pulmonary edema.

ECMO has been used in patients with severe HCPS in the cardiopulmonary phase who are judged to be at high risk of death (175). In a series of patients treated with ECMO between 1994 and 2010 who had predicted mortality of almost 100% without ECMO, overall survival was 66%. Furthermore, survival was 80% among 25 patients treated between 2003 to 2010 when the group practiced elective insertion of vascular sheaths with almost concurrent intubation and initiation of ECMO when the patients became unstable. Criteria for ECMO include a cardiac index of less than 2.31 per min per square meter, or an arterial oxygen tension to fractional inspired oxygen (PaO_2/FIO_2) ratio of less than 50, and lack of response to conventional (non-ECMO) support (148).

Shortly after recognition of HCPS in the United States in 1993, open-label intravenous ribavirin was available for persons with suspected HCPS through a CDC-sponsored protocol. Therapy was considered justified because it had reduced mortality in hantavirus-associated HFRS in a controlled trial and because all previously tested hantaviruses had been sensitive to ribavirin *in vitro* (176, 177). Among 140 persons suspected of having HCPS who received ribavirin, HCPS was confirmed in 30 and ruled out in the remaining 110 (178). Among the 64 patients diagnosed in the United States during the study period, death occurred in 14 (47%) of 30 patients treated with ribavirin as compared to

17 (50%) of 34 who were not enrolled in the treatment study. Ribavirin-associated toxicity included reversible anemia in 71% of recipients; 19% required transfusions (178). Adverse events that were less clearly related to ribavirin therapy included pancreatitis or hyperamylasemia that were documented in 8% of patients.

A subsequent, prospective, placebo-controlled trial of intravenous ribavirin for suspected HCPS in the United States and Canada, sponsored by the National Institute of Allergy and Infectious Diseases Collaborative Antiviral Study Group, failed to reach its target accrual and was closed based on a futility analysis (179). However, there were no trends suggesting a reduction in mortality in patients enrolled during the cardiopulmonary phase (Figure 8), and the investigators were unable to enroll subjects in the febrile prodrome. The median time to progression to death or initiation of ECMO was 24 hours or less in both treatment groups, suggesting that there is a very limited window of opportunity for intervention once patients present in the cardiopulmonary phase (Figure 8) (179).

Studies suggested benefit from intramuscular cortisone treatment of HFRS during the Korean conflict, and observational studies suggesting benefit from high-dose intravenous methylprednisolone for HCPS in Chile (180, 181). A randomized double-blind, placebo-controlled trial in Chile assessed high-dose intravenous methylprednisolone treatment of HCPS in the cardiopulmonary phase (182). Among the 60 with confirmed ANDV infection, there was no significant difference in progression to primary endpoints, including shock, respiratory failure, or death. Furthermore, the risk of death was determined by the severity of illness at study entry, whereas methylprednisolone treatment had no impact on survival after adjustment for disease severity at entry (Figure 9).

More recently, investigators in Chile conducted an open trial of human immune plasma with an ANDV neutralizing antibody (NAb) dose of 5,000 U/kg for treatment of HCPS (183). Donors were plasmapheresed at least 6 months after recovery from HCPS, and ANDV NAb titers were measured by a focus reduction assay. Low case fatality rates, 14/29 (14%) were low, but efficacy could not be evaluated in this nonrandomized, open trial. Future studies should ideally be performed with a product without the need for blood type compatibility, NAb directed against both SNV and ANDV, and feasibility of development of a commercial product (183).

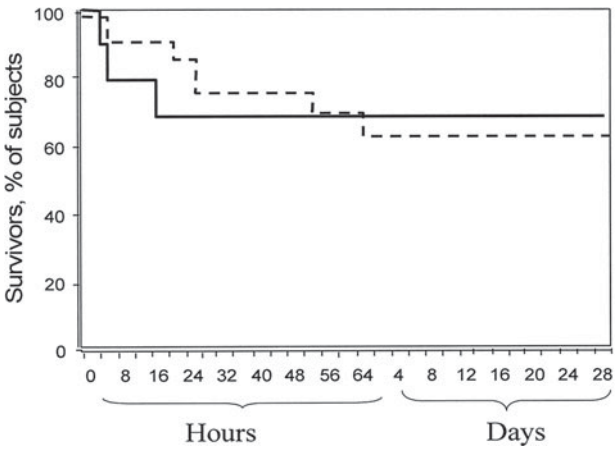
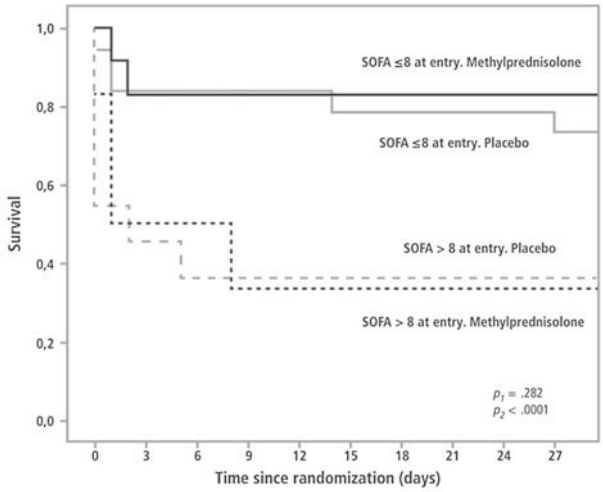


FIGURE 8 Survival without extracorporeal membrane oxygenation among ribavirin recipients versus placebo recipients in a trial of intravenous ribavirin therapy for hantavirus cardiopulmonary syndrome. Solid line, ribavirin recipients (N = 10); broken line, placebo recipients (N = 13). Reprinted from reference (179) with permission of the publisher.



Number at risk

Methyl prednisolone	30	27	23	22	22	15	22	22	22	22
Placebo	30	21	20	20	20	20	19	19	19	18

FIGURE 9 Kaplan-Meier survival analysis by treatment arm and severity at entry. *P*1: significance between methylprednisolone and placebo arm. *P*2: significance between sequential organ failure assessment (SOFA) >8 and ≤8 at entry. Reprinted from reference (182) with permission of the publisher.

Hemorrhagic Fever with Renal Syndrome

Management of HFRS should include monitoring for shock, hypotension, renal failure, and hemorrhage. Whenever possible, both critical care facilities and hemodialysis should be readily available. Comparison of case fatality rates before and after the availability of hemodialysis and critical care suggest that mortality rates have been significantly reduced by the availability of the latter (136, 151–153).

A prospective, placebo-controlled trial of high-dose intravenous ribavirin in patients with serologically confirmed hantavirus-associated HFRS in the People's Republic of China has established the value of this intervention (176). Nonpregnant patients 14 years of age or older with fever and proteinuria with a significant exposure history or the presence of hantavirus-specific IgM antibodies were eligible for enrollment unless they had had symptoms for more than six days, advanced renal failure, coma, or shock refractory to fluid replacement. Patients who received ribavirin received a loading dose of 33 mg/kg followed by 16 mg/kg every 6 hours for 4 days and 8 mg/kg every 8 hours for 3 days.

Of 242 patients with serologically confirmed hantavirus-associated HFRS who were included in the efficacy analysis, there was a 7-fold decrease in the risk of mortality among ribavirin-treated patients (P = 0.01) after adjustment for baseline predictors of mortality (176). Ribavirin therapy also significantly reduced the risk of developing oliguria and hemorrhage. The primary adverse effect of therapy was hemolytic anemia that resolved after discontinuation of therapy. The proportion of patients with severe anemia (hematocrit below 20) was similar in the two treatment

groups. Development of severe anemia was gradual in the ribavirin-treated patients, whereas placebo-treated patients with severe anemia tended to develop acute drops in hematocrit secondary to gastrointestinal or urinary tract hemorrhage.

A similar, placebo-controlled trial, also conducted in the People's Republic of China, evaluated the efficacy of treatment with alpha-interferon at doses of 1×10^7 units per day. Although the risk of hemorrhage was reduced with treatment, treatment with interferon did not reduce mortality (184). Recently, another approach was suggested to interact with the pathogenesis, i.e., to inhibit the bradykinin receptor with an antagonist, icatibant, and thereby decrease the capillary leakage syndrome. One severe case treated using this method survived but further studies are needed (185).

PHLEBOVIRUSES

Rift Valley Fever

Epidemiology

Rift Valley fever is endemic in Africa and the Arabian Peninsula, with major outbreaks in Egypt in 1977 and 1993, in Yemen in 2000, in Saudi Arabia in 2000 to 2001, and in Kenya in 2007 (186–189). Epizootics, particularly in sheep and cattle, and epidemics may occur after periods of high rainfall and flooding, as well as following the introduction of irrigation projects. During outbreaks, the economic consequences, including the ban of animal trade and loss of livestock, may be devastating for the rural communities in endemic countries.

Transmission of RVF virus is by insect vectors for epizootics and for most human infections by direct contact with infectious tissues or blood from abortion or slaughter of infected animals (189, 190). The virus has been isolated from several genera of mosquitoes and from sandflies, suggesting that multiple insect vectors may be important in maintaining epizootic disease. In the absence of epizootic disease, the virus can be maintained for years during drought through transovarial transmission of virus (particularly in *Aedes mcintoshi*), persistence of infected viable mosquito eggs in soil, and reemergence of infection after rainfall and hatching of the infected eggs (7, 191). Also, an enzootic cycle of the virus may be present in wild animals (192). Amplification in sheep, cattle, and goats would appear to be more important than transmission between mosquitoes. The infection has a mortality rate of 10 to 30% in sheep, cattle, and goats and is associated with abortion and almost 100% mortality in pregnant animals.

Humans at risk include persons residing in rural, agricultural areas where an epidemic or epizootic is recognized, persons with exposure to areas where deaths and abortions are occurring in livestock, particularly if they perform necropsy, and those with exposures in laboratories or abattoirs. In an analysis of an outbreak of RVF in Kenya in 1997 to 1998, contact with sheep body fluids and sheltering livestock in one's home were significantly associated with infection (193). Although humans can be infected from bites of mosquitoes and other arthropod vectors, contact with viremic animals, particularly livestock, appears to be the most common mode of acquisition. This is supported by multivariate analyses of risk factors for infection, which include contact with sheep body fluids and sheltering livestock in the home, as well as by relatively low rates of infection in young children (193, 194). The disease occurs in all age groups and in both sexes. Men seem to have an increased risk, presumably through agricultural activities and animal contact. In the large outbreaks in East Africa in 2007, more male than female cases were recognized (195–197). Since the RVFV has a broad vector competence, there is a rising concern that it may spread to Europe and the Americas similar to West Nile and Chikungunya viruses (198, 199).

Pathogenesis

The incubation period ranges from two to six days (7, 189, 190). Viremia occurs in domestic animals and in humans during the acute illness. In animals, target organs include the liver and brain, and in sheep, death usually results from hepatic necrosis. Deaths in humans most commonly occur among those who develop a hemorrhagic fever, shock, and liver necrosis during the acute episode, and virus can be detected or isolated from postmortem blood or liver samples (7, 64, 189, 190, 200, 201). In contrast, virus cannot be isolated from persons with the late clinical complications (encephalitis, retinitis, and uveitis). The time course, lack of viremia, and presence of virus-specific antibodies all suggest that these late complications may be immune-mediated. Animal models for both the acute hepatic necrosis and the late encephalitis are available.

Clinical Manifestations

Infection may be asymptomatic, mild, or cause severe disease with substantial morbidity and mortality (193, 202). Most patients with Rift Valley fever (RVF) develop a benign febrile illness with fever, myalgia, and malaise (189, 190, 200, 202–204). Symptoms typically resolve in two to five days but may be followed by a prolonged convalescent period.

The overall mortality of RVF has been estimated to be 1% or less (194). In approximately 1 to 3% of patients the infection presents as a more severe and life-threatening viral hemorrhagic fever; among hospitalized patients with confirmed RVF, both mortality and complications have been reported at much higher frequency. Among 165 patients hospitalized at one hospital in Saudi Arabia in 2000, hepatic failure occurred in 75%, acute renal failure in 41%, and hemorrhagic complications in 19%. Hepatorenal failure, shock, and severe anemia were major factors associated with death among the patients who died (186).

Mortality is also much higher than 1% in case series, probably because of underreporting of mild cases in the case series. In an analysis of 834 reported cases of RVF in Saudi Arabia in 2000 to 2001, of which 81% were laboratory confirmed, the overall mortality rate was 13.9%. Bleeding, neurologic manifestations, and jaundice were independently associated with increased mortality, and patients with leucopenia had lower mortality than patients with normal or increased leukocyte counts (188). Similarly, a case-fatality rate of 29% was reported among 404 probable or confirmed cases of RVF in Kenya between November 2006 and January 2007 (194).

Patients with encephalitis often have a biphasic illness, with onset of headache, nuchal rigidity, confusion, hallucinations, coma, or focal neurologic findings one to four weeks after onset of RVF.

Ocular complications of RVF have long been recognized as a serious but relatively uncommon complication (203). In a cross-sectional study in Saudi Arabia, 165 eyes in 113 outpatients and 47 eyes in 30 inpatients were affected with an interval of 4 to 15 days (mean 8.8 days) between onset of symptoms of RVF and onset of visual symptoms (205). Macular or perimacular retinitis was identified in all affected

eyes, ranging from retinal hemorrhage to optic disc edema and retinal vasculitis. Transient anterior uveitis was present in 31% of cases. Visual acuity was less than 20/200 in 80%, and vision remained the same in 72% and deteriorated in 15% of affected eyes over time. Permanent visual loss was associated with macular and perimacular scarring, vascular occlusion, and optic atrophy.

Laboratory Diagnosis

Detection of viral antigen and virus-specific IgM antibodies are the preferred diagnostic tests during acute illness, although detection of IgM antibody in the absence of IgG antibody should be interpreted with caution. The presence of only IgG could be due to a previous infection, so convalescent serum with 4-fold increase of IgG titer is required to verify the diagnosis. Serologic diagnosis may be accomplished with a single convalescent serum using IgM-capture ELISA (or less reliably by IgM-IFA) or with comparison of acute and convalescent sera by neutralization, HI, IFA, or ELISA.

Virus isolation is straightforward, provided blood is obtained during the acute illness, and BSL-3 facilities are available (189–191, 200, 202, 204). Sensitive isolation systems include Vero or AP61 mosquito cells, intracranially inoculated suckling mice, or intraperitoneally inoculated adult mice or hamsters. The virus grows efficiently in many cell lines, is cytopathic, and plaques easily.

As for other viral hemorrhagic fever viruses, the diagnostic use of qualitative or quantitative RT-PCR is safer and more efficient than viral culture. Neither virus isolation nor antigen or nucleic acid detection is helpful in patients with late complications like encephalitis or ocular disease, but a presumptive diagnosis can be established based on rising serum IgG titers and through detection of specific IgM in serum and, occasionally, in CSF.

Prevention

As one mode of transmission of Rift Valley fever virus is via mosquito bites, the risk of infection could presumably be reduced by careful use of mosquito repellents, particularly during periods of high rainfall and high mosquito density when the risk of epizootics is most marked. The risk of direct transmission from animals during handling, slaughter, autopsy, or disposal is greatest during epizootics, and particular care should be taken at this time. The presence of an epizootic should be suspected in the setting of unexplained deaths of lambs and sheep and abortions in ewes in an area that is known to be at risk for RVF (202).

Early case recognition by physicians and veterinarians together with surveillance of animals and vector control are important to mitigate outbreaks. This approach has been suggested as the "One Health" concept, which combines the efforts of human and animal health together in the prevention of zoonotic infectious diseases (206).

No special precautions are indicated when caring for infected persons or when handling blood or other tissues beyond those routinely used to prevent nosocomial transmission of blood-borne infections. However, when the differential diagnosis could potentially involve diseases such as CCHF, Lassa fever, or Ebola infection that have substantial risk of nosocomial transmission, strict isolation is indicated until RVF is diagnosed or the patient recovers.

Currently, there is no approved human vaccine (207, 208). Live attenuated vaccines have been used to prevent Rift Valley fever in animals, especially during outbreaks. They are not safe enough to be used in humans, and the live attenuated vaccine can induce abortion in animals. Less reactogenic clones of RVFV vaccine have been evaluated in animal trials, but the issue of reversion to more virulent strains remains. The technique of reverse genetics has enabled researchers to delete genes associated with virulence and potentially create safer vaccines (208–210).

Both inactivated and attenuated vaccines have been tested for their ability to prevent infection in sheep, cattle, goats, and other animals both to prevent epizootics and economic loss and to prevent human infections. Control measures in a recent outbreak in Kenya included a ban on slaughter of livestock, closure of livestock markets, and use of a live attenuated vaccine for animals (194).

Treatment

There is no specific therapy for Rift Valley fever, for the hemorrhagic complications or for the late complications of encephalitis and retinitis. Parenteral ribavirin therapy is effective in preventing or significantly reducing the level of viremia in a rhesus monkey model, and alpha or purified human leukocyte interferon therapy is effective in the same model (211–213). Passive antibody and interferon inducers are also effective in animal models. No human trials have been reported for prevention or treatment of RVF, and there are no current guidelines for passive immunoprophylaxis or antiviral chemoprophylaxis of RVF. Thus, early treatment with ribavirin, alpha or human leukocyte interferon, or passive neutralizing antibody might reduce the risk of complications, but no trials have been performed in humans. Systemic corticosteroids have been used in patients with retinal vasculitis and encephalitis without apparent benefit. The RNA polymerase inhibitor, favipiravir (T-705) has antiviral activity against RVFV *in vitro* and in animal models (214, 215).

Sandfly Fever Viruses

Epidemiology

Phlebotomus fever viruses are found in both hemispheres (6, 7). Sicilian and Naples viruses, transmitted by phlebotomine sandflies, continue to cause human disease in countries bordering the Mediterranean Sea. Residual spraying of walls in homes has been particularly successful, since the vectors feed primarily at night within buildings and tend to rest on building walls, but sandflies may penetrate mosquito netting. Most human infections occur in late spring, summer, and early fall. Epidemics have been described, particularly during wartime, including epidemics in Italy during the Second World War (216). The flight range of the sandfly is limited, so attack rates may vary considerably during epidemics. Toscana virus, which is transmitted by *P. perniciosus* and other sandflies, is a common cause of aseptic meningitis and meningoencephalitis in the Mediterranean basin (217, 218).

The viruses are presumably amplified in wildlife vertebrate hosts, but these have not been identified. Transtadial and transovarial transmissions in sandflies have been demonstrated and are probably the primary mechanisms of perpetuation, particularly in the desert (219, 220).

New World phleboviruses, including Chagres, Punta Toro, and Alenquer, have been isolated during sporadic febrile illnesses from humans in Panama and Brazil who work or live in jungle areas. No epidemics have been identified, and the sandfly vectors appear to be limited to forests.

Pathogenesis

In volunteers, the incubation period ranges from two to six days (216). Infected humans are viremic during the acute

febrile illness, and Toscana virus has been isolated from CSF of patients with aseptic meningitis and meningoencephalitis (217, 218, 221–223). No deaths have been reported. Neutralizing antibodies persist after infection, and immunity is probably lifelong.

Clinical Manifestations

Most infections with sandfly fever viruses appear to result in clinical disease, but mild or subclinical infections may be more common in children. After an incubation period of two to six days, the patient develops a febrile illness that lasts two to four days (216). Toscana virus causes aseptic meningitis and meningoencephalitis (217, 218, 221–223), but high seroprevalence rates in central Italy suggest that most persons infected with this virus either have subclinical infection or a clinical syndrome other than aseptic meningitis (224). No deaths have been reported with these viruses, but neurologic sequelae including deafness and personality changes may develop (225, 226).

Laboratory Diagnosis

Virus isolation is easily achieved from blood obtained during the three-day fever; the viruses replicate well, are cytotoxic, and form plaques in Vero cells (191, 216, 219, 220, 227). Toscana virus has been isolated from CSF and may also be detected by RT-PCR (218, 228). Serologic diagnosis can be performed with IGM-IFA, or preferably, by IgM-capture ELISA shortly after resolution of the fever or by serology on paired sera using techniques such as ELISA, HI, or plaque reduction neutralization (228).

Prevention

Infection with sandfly fever virus can best be avoided by use of insect repellants containing DEET. No special isolation requirements are necessary for persons with sandfly fever. Although oral treatment with 1,200 mg/day of ribavirin prevented viremia and clinical manifestations in volunteers inoculated with Sicilian sandfly fever virus, this approach has not been evaluated during a natural epidemic (213). No specific therapy is available for patients with symptomatic infection. Investigational pyrazine derivatives, including T-705 (favipiravir), have shown activity against Naples virus *in vitro* and in animal models (214).

Severe Fever with Thrombocytopenia Syndrome

Epidemiology

Severe fever with thrombocytopenia syndrome (SFTS) is caused by a novel tick-borne phlebovirus, SFTS virus, which was first recognized in China during 2007 to 2009 (1, 229). The disease had been reported from at least 11 provinces in China, and cases have also been detected in Korea and Japan (1). A similar disease caused by Heartland virus, which is spread by *Amblyomma americanum* (lone star tick), has also been detected in the United States (2).

Among 171 patients fulfilling the case definition of SFTS (acute fever of unknown cause, temperatures of 38°C or more, and thrombocytopenia), 154 were laboratory confirmed as SFTS (232). Most of the patients with SFTS have been farmers, living in wooded and hilly areas that had been exposed when working in the fields. The virus has been found in *Haemaphysalis longicornis* ticks collected from domestic animals in the same area as the cases reside (229). More cases occur in spring and summer when persons are out in the field and more exposed to ticks. Slightly more women (55 to 57% of cases) have been affected (218, 230). Among 504 confirmed cases in the most affected region, Xinyang, the vast majority have been older farmers (median age 61 years) living in rural areas (231). Environments with risk include shrub, rain-fed cropland, and forest areas. Seroprevalence studies from affected counties in China show that 1 to 3.8% of the populations in endemic areas have specific IgG antibodies toward SFTS virus (1).

Pathogenesis

Both the level of viral replication and the host immune responses likely play roles in the pathogenesis of SFTS. Higher blood viral load when the patient is admitted to the hospital and when sustained during the second week of illness is associated with fatal outcome (232). Viremia may last several weeks after onset of illness (232). The infection leads to elevated levels of cytokines, chemokines, acute phase proteins, and coagulation disturbances, which are correlated to viral RNA levels. SFTS viral RNA can be detected not only in blood, but also in the upper respiratory tract, urine, and feces (232). The infection leads to an innate immune response with elevated natural killer cells and increased plasma cytokine levels, e.g., G-CSF, IP-10, MCP-1, IL-1RA, IL-6, and IL-10 (233, 234). The cytokine levels seem to correlate to the severity and outcome of the infection (234). In contrast, the nonstructural protein, NSs, and the nucleocapsid protein both encoded by the S segment may suppress the activation of beta-interferon and NF-κB (235).

A mouse model for the pathogenesis of SFTS has been established and showed similar pathological changes as in humans, i.e., thrombocytopenia and leukopenia in the infected mice (236). Histopathological changes and viral RNA were identified in several organs, the liver, kidney, and spleen. Similarly, infection with SFTS virus in rhesus macaques was characterized by fever, thrombocytopenia, leukopenia, and increased levels of liver and myocardial enzymes but less severe symptoms (237).

Clinical Manifestations

The most prevalent symptoms in SFTS are fever, myalgia, anorexia, fatigue, nausea, and gastrointestinal symptoms (229, 238). Common laboratory findings are thrombocytopenia, leukopenia, elevated levels of lactate dehydrogenase (LDH), serum aspartate aminotransferase (AST), alanine transaminase (ALT), and creatine kinase (238). Severe cases may have disturbance of consciousness, acute respiratory distress syndrome, hemorrhagic signs, coagulopathy, renal function impairment, and arrhythmia. Disseminated intravascular coagulation may lead to organ dysfunction, multiorgan failure, and death (229, 239, 240). Patients with older age, decreased level of consciousness, elevated levels of lactate dehydrogenase, and creatine kinase have significantly higher risk for fatal outcome (238). The case-fatality rate is 12 to 30% in China (230, 231, 238, 240).

Many of the symptoms presented in SFTS are nonspecific and there are several differential diagnoses, for instance hantavirus, dengue, and infections caused by leptospira and rickettsia. Therefore, an adequate case definition together with fast and reliable diagnostic methods are important. In South Korea, using a case definition including fever (body temperature >38.0°C), leukocytopenia, thrombocytopenia, and symptoms from the gastrointestinal tract, 35 patients were identified in a hospital-based surveillance during 2013 (241).

Laboratory Diagnosis

The diagnosis of SFTS is based on detection of specific antibodies, viral antigen, or viral RNA by RT-PCR (241).

The virus can be cultivated on Vero E6 and other cells, but culture takes time and could be hazardous. The virus should be handled at Biosafety level 3 laboratories. For detection of contagious and potentially lethal Bunyaviruses, RT-PCR is recommended instead of culture since the virus is killed off during RNA extraction, providing a rapid, safe, and specific method for diagnosis of SFTS in viremic patients with disease (242–244).

Serological testing is done by detection of specific IgM and IgG antibodies by ELISA, using recombinant nucleocapsid protein of SFTS virus (229, 245, 246). Antibodies develop after 7 days after disease onset. If only IgG is analyzed, seroconversion, or a 4-fold raise of titer, is required for diagnosis of STFS, especially in endemic areas. In addition to diagnosis of acute SFTS patients, an ELISA method can also be useful to study the prevalence of the infection among humans and animals (245, 246).

Prevention

There is no available vaccine for SFTS virus infection. Prevention is based on avoiding ticks and tick bites in endemic regions. Insect repellents that are effective against ticks may be used for prevention of several tick-borne infections. When bitten, ticks should be removed as quickly as possible. It has been recommended that patients should be isolated during the acute and viremic phase (1). When handling blood and other specimens, gloves and other protective clothing should be used.

Treatment

The case fatality rate may be as high as 30%, and there is no effective antiviral treatment. Symptomatic treatment, supportive care, and fluid replacement therapy can decrease the risk for shock and fatal outcome. The antiviral ribavirin appeared ineffective when used for SFTS patients (238, 241). In one nonrandomized, observational study of 311 SFTS patients, there was no difference in case fatality rates (CFR) between 138 patients that received ribavirin (CFR 17.4%) compared with 164 who did not receive ribavirin (CFR 17.1%). Furthermore, ribavirin had no significant effect on viral loads or platelet counts (238). A promising approach and alternative treatment may be human neutralizing monoclonal antibodies toward SFTS virus, which is inhibitory *in vitro* (247).

NAIROVIRUSES

There are at least 34 viruses in the genus *Nairovirus*, all of which are tick-borne, which have been classified in seven serogroups (248–250). The latter included the CCHF group, which include the human pathogens CCHFV and Hazara virus, and the Nairobi sheep disease (NSD) group includes the human pathogens NSD virus (NSDV) and Ganjam virus. Ganjam virus, which has been isolated from ticks collected from goats in India and has been associated with febrile illness in humans in India, appears to be an Asian variant of NSD virus (250). Dugbe virus infection was described in a patient who suffered from a febrile illness with prolonged thrombocytopenia in South Africa (251).

Crimean-Congo Hemorrhagic Fever

Epidemiology

Crimean-Congo hemorrhagic fever (CCHF) virus is distributed over the geographic range of *Hyalomma* ticks and includes Africa, Eastern Europe, the Middle East, and Asia through the western provinces of China (Figure 10) (7, 33, 248, 252). There is little evidence for subclinical infection, and most humans are seronegative in endemic areas.

Infection occurs through tick bites or by direct contact with infected animals or humans or their tissues. Cows, sheep, goats, hares, and other herbivores have all been implicated in transmission and amplification of CCHF virus. While birds are not susceptible to CCHF virus infection, many *Hyalomma* species feed on birds, thereby allowing the migrating birds to play an important role in disseminating CCHF virus–infected ticks. Antibody to CCHF virus has been found in 28% of cattle sera and 78% of cattle herds tested in South Africa and in 45% of cattle sera and 94% of herds tested in Zimbabwe (252). CCHF appears to be maintained both by transovarial and transtadial cycles in ticks and by a tick-vertebrate cycle. In temperate climates, peak transmission generally occurs in the spring and summer when populations of ticks and their vertebrate hosts peak.

The risk of infection in humans after a bite from a CCHF-infected tick is not known but is presumed to be high. Secondary cases have occurred both in laboratory workers and in hospital workers with direct contact with patients or their blood and respiratory secretions (253–255). There are multiple reports of nosocomial transmission, including one in which infection occurred in 8.7% of healthcare workers exposed to blood and in 33% of those with a needlestick injury (248, 256). Viremia is highest during the first three days of illness and may persist through the second week (257). Tertiary cases have also been described in family contacts of healthcare workers with CCHF.

Pathogenesis

The pathogenesis of CCHF is less well understood than for many of the other hemorrhagic fevers, in part due to the lack of an appropriate animal model. Viremia is present during the illness, and titers are highest during the first three days of illness (257). Disseminated intravascular coagulation (DIC) is present, particularly in the more severe cases (258). Fatal infections are also heralded by markedly elevated serum aspartate amino transaminase, alanine amino transaminase, and creatine kinase and, terminally, by elevated bilirubin, creatinine, and BUN. Clinically, multiple organ failure precedes death, with involvement of the brain, liver, kidneys, lungs, and heart. Haemophagocytosis, which may be associated with high levels of Th1 cytokines, has been reported in severe CCHR, and levels of pro-inflamatory cytokines IL-6 and TNF alpha and DIC scores are higher in patients with fatal versus nonfatal CCHF (259, 260). Of note, a CCHF virus glycoprotein precursor is cleaved to generate a novel glycoprotein that is similar to an Ebola virus domain associated with increased vascular permeability and development of hemorrhages (261).

At autopsy, edema and focal hemorrhage and necrosis are present in multiple organs, including the liver and brain. Although focal hepatic necrosis is most common, massive hepatic necrosis has also been described (258). Focal hemorrhage and necrosis may be found in the brain, and herniation may occur. Renal lesions are characterized by focal, tubular necrosis.

Clinical recovery is temporally associated with resolution of viremia and with appearance of specific IgG and IgM antibodies, generally between days 7 and 9 of illness. In contrast, endogenous antibody production was demonstrated in only 2 of 15 patients who died of CCHF (262). There is little information regarding the role of cell-mediated immune responses or the role of nonspecific defenses such as natural killer cells or cytokines.

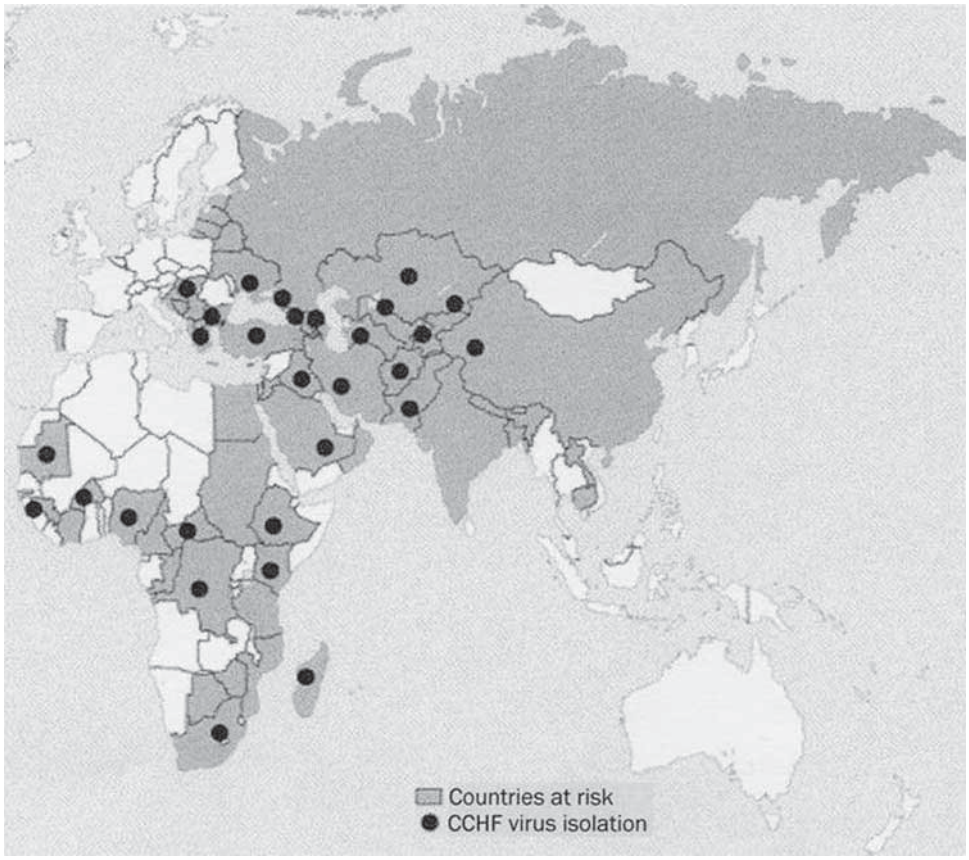


FIGURE 10 Worldwide distribution of CCHF virus. Reprinted from reference (248) with permission of the publisher.

Clinical Manifestations

The incubation period ranges from three to six days in nosocomial transmission, whereas after exposures to animals or ticks it ranges from two to twelve days (56, 190, 252, 253). The illness typically begins as an acute febrile illness with fever, chills, myalgia, headache, and nausea, with or without abdominal pain and emesis (252, 253, 258, 262). During this stage, patients are often flushed and have injected conjunctivae or chemosis. After three to six days of illness, often after a brief period of clinical improvement, most patients develop hemorrhagic manifestations (Figure 11) (248). These may be limited to petechiae over the trunk and limbs or may involve large ecchymoses. Epistaxis, hematemesis, melena, and hematuria are common during this phase. Both hepatomegaly (20 to 40% of cases) and splenomegaly (14 to 23%) may be present (248). Patients may be somnolent, and dizziness and mild meningeal signs are common. Beginning at about the fifth day of illness, the most severely ill patients develop disseminated intravascular coagulation. Some series have emphasized hepatorenal failure, bradycardia, and hypotension in fatal cases, but this was not reported in recent series in Turkey (248).

Patients who survive have rapid resolution of fever and hemorrhagic manifestations that may be followed by a convalescent stage characterized by persistent fatigue. Poor prognostic indicators include overt DIC; hematemesis, melena, and somnolence; AST and ALT levels >700 and >900 IU/l, respectively; platelet counts below 20,000 and low platelet counts (below 50,000 cells/ml) that fail to increase over the first few days of hospitalization (248). The case-fatality rate has averaged about 30%, but has ranged from 10% to more than 64% (248); the case-fatality rate may be greater in nosocomial cases. In a series from South Africa from 1981 to 1987, 15 of 50 patients died between days 5 to 14 of illness (258, 262). The proportion of subclinical infections is not known, but one model suggested that CCHF developed in 20% of infected persons (263).

Clinical laboratory abnormalities are helpful in suggesting the diagnosis, and marked elevations in hepatic transaminases, thrombocytopenia, and findings consistent with severe DIC during the first few days of illness are of prognostic value. Leukopenia is usually present from the onset of illness in nonfatal cases, whereas granulocytosis is often present during the first week in fatal infections. Thrombocytopenia is uniformly present early in the course of the illness in fatal cases and appears to be universally present by the end of the first week of illness. DIC occurs in both fatal and nonfatal infections, but abnormal activated partial thromboplastin times (APTT), thrombin times (TT), and fibrin degradation products (FDP) occur earlier and are more abnormal in fatal as compared to nonfatal cases (258).

Before the development of hemorrhagic manifestations, the differential diagnosis would include any cause of febrile illness. Many patients will have an occupational or rural exposure history. Depending on the patient's location and recent travel history, the differential diagnosis could include leptospirosis, brucellosis, septicemic plague, malaria, tularemia, tick typhus, Rift Valley fever, or sandfly fever. Once hemorrhagic manifestations develop, the differential diagnosis should include other hemorrhagic fevers that are

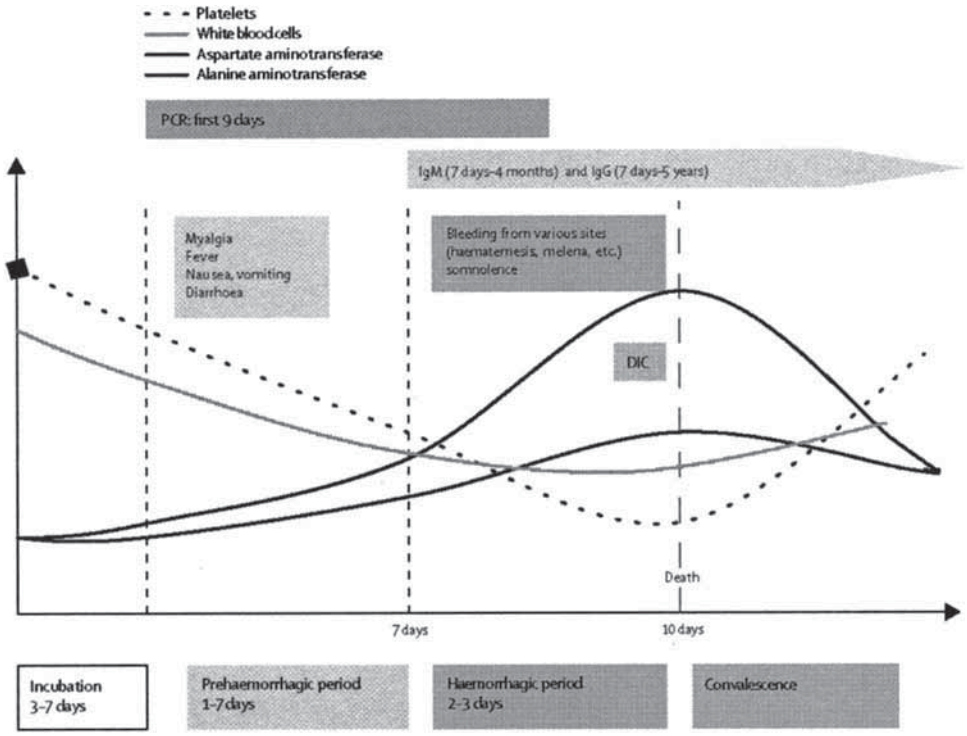


FIGURE 11 Clinical and laboratory course of CCHF. Reprinted from reference (248) with permission of the publisher.

known to be present in the region. These may include Kyasanur Forest disease, Omsk hemorrhagic fever, HFRS, Lassa fever, yellow fever, Ebola, or Marburg virus infection, and dengue hemorrhagic fever or dengue shock syndrome. If nosocomial transmission occurs following an index case with an undiagnosed hemorrhagic fever, the differential diagnosis would be limited to known, regional causes of hemorrhage and to agents that are known to be transmitted nosocomially. The latter include CCHF virus, SFTS virus, Lassa fever virus, Marburg virus, and the Ebola viruses.

Laboratory Diagnosis

CCHF virus can be easily isolated from blood during the acute illness, particularly during the first three days when virus titers are greatest (252, 253, 257, 258, 262), but should only be attempted in BSL-4 facilities. After intracranial inoculation of suckling mice, the mice die in four to eight days, and the diagnosis can be established by CF or FA testing of brains. Primary isolation can also be performed in cell culture and confirmed by detection of viral antigen by FA once cytopathic effect is seen. Comparison of primary isolation in suckling mice and in chicken embryo related (CER) cells or Vero E6 cells suggests that the former is somewhat more sensitive (257).

The method of choice for rapid viral diagnosis is real-time reverse transcriptase PCR (RT-PCR), which is highly sensitive and specific (264). An antigen-capture ELISA, which is less sensitive, in one series detected antigen in most fatal cases and approximately half of nonfatal cases (265).

Serodiagnosis can be made using acute and convalescent titers or by detection of IgM antibody in early convalescence. Serologic assays are usually negative during the first week of illness and in persons who die from CCHF. The relative sensitivities of serologic assays in a series of 35 survivors of CCHF (262) were ELISA, reversed passive hemagglutination-inhibition (RPHI), IFA, fluorescent-focus reduction, CF, and immunodiffusion in decreasing order. IgG antibodies became detectable by IFA on days 7 to 9 in the 35 survivors; IgM antibody was present in one patient on day 5, and was usually present one day before or after the first detection of IgG antibody. A neutralization test is also available in some research centers.

Prevention

The risk of tick bites can be reduced by use of permethrin-impregnated clothing, by frequent inspection for the presence of ticks, and by gentle removal of ticks (without crushing the tick) if tick bites occur (266). Repellants such as DEET are relatively ineffective against ticks, but application of acaracides to domestic animals may be helpful. In light of the numerous examples of secondary transmission to healthcare workers, and sometimes to their own family contacts, strict isolation should be followed when caring for a patient with known or suspected CCHF. Recommendations for veterinarians, abattoir workers, and others who may acquire CCHF from direct contact are less clear, but presumably would include avoidance of contact with blood and body tissues, particularly with animals that appear sick.

A formalin inactivated–suckling mouse-brain-derived vaccine has been evaluated for immunogenicity in humans but appears to elicit only very low neutralizing antibody titers. There are no controlled data regarding antiviral chemoprophylaxis, but limited *in vitro* and animal data suggest that oral or intravenous chemoprophylaxis with ribavirin might be effective. In a nosocomial outbreak of CCHF at the Tygerberg hospital in South Africa, six of nine persons with needle-stick exposure were given prophylactic ribavirin. One of the six treated persons developed a mild clinical disease and the remainder remained clinically well and seronegative (256, 267). While this limited experience does not prove

efficacy, this approach might be considered following a high-risk exposure in the laboratory or in a healthcare setting. Close follow-up for 14 days with administration of ribavirin if fever develops is another strategy (248).

Treatment

Aggressive, supportive therapy, preferably in a setting with critical care capabilities, is essential for patients with this serious hemorrhagic fever. Hemorrhage can lead to large requirements for blood replacement with attendant complications. Replacement of platelets and coagulation factors with fresh frozen plasma may be indicated, and shock and less severe intravascular volume disturbances may necessitate invasive monitoring.

There is no proven, specific antiviral therapy for CCHF in humans. Ribavirin is active *in vitro* and in an infant mouse model of CCHF (268, 269). No controlled trials of ribavirin have been reported in humans. However, several open series have documented low mortality rates in persons with CCHF treated with ribavirin when compared with historical controls (254, 270), and nonrandomized case series also suggest that oral ribavirin treatment reduces mortality (271, 272). If intravenous ribavirin is available, the recommended regimen is a 30 mg/kg loading dose followed by 15 mg/kg every 6 hours for 4 days followed by 7.5 mg/kg every 8 hours for 6 days; we recommend maximum intravenous doses of 2 g, 1 g, and 500 mg, respectively (179, 248). IV administration of specific immunoglobulin for CCHF virus has also been reported in uncontrolled trials (273, 274). Notably, the new compound favipiravir (T-705) showed better efficacy than ribavirin in a lethal mouse model for CCHF (275).

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Arenaviruses

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Viruses of the *Arenaviridae* family (genus *Arenavirus*) are zoonotic; they are maintained in nature, with a few possible exceptions, by chronic infection in rodents of the superfamily Muroidea (1). Over 40 arenaviruses have been identified, although less than half of these are clearly recognized as human pathogens (Table 1 and Figures 1 and 2). Arenaviruses continue to be discovered at a quickening pace in recent decades (2–10).

Arenaviruses are perhaps best known as agents of viral hemorrhagic fever (VHF), an acute systemic illness classically involving fever, a constellation of initially nonspecific systemic signs and symptoms, and a propensity for causing bleeding and shock (11, 12). Similar VHF syndromes are caused by members of the virus families *Bunyaviridae*, *Filoviridae*, and *Flaviviridae* (see chapters 9, 42, 44, and 53). A few arenaviruses have been associated with aseptic meningitis and other central nervous system (CNS) diseases, including congenital malformations when transmitted *in utero*. Many arenavirus infections are asymptomatic or result in a nonspecific febrile illness difficult to distinguish from many more common diseases.

Seven arenaviruses have been clearly established as causative agents of VHF via natural infection (i.e., excluding laboratory infections): Lassa and Lujo in Africa, and Junín, Machupo, Guanarito, Sabiá, and Chapare in South America (Table 1). Two Old World arenaviruses, lymphocytic choriomeningitis virus (LCMV) and an LCMV variant named Dandenong virus, are typically associated not with VHF but rather with febrile illness and CNS disease (13). Whitewater Arroyo virus has been detected in three sick persons in California but its role as a pathogen remains to be confirmed. Tacaribe, Pirital, and Flexal viruses have caused nonspecific febrile illnesses after laboratory accidents but no natural infections have been recorded. Pichinde and Pirital viruses are frequently used in animal models (guinea pig and hamster, respectively) of arenaviral hemorrhagic fever.

Because of their potentially high lethality, risk of secondary spread (although this is often overestimated), and tendency to cause public panic and social disruption, some VHF-causing arenaviruses are considered "Select Agents" that could possibly be used as bioweapons. Attempts to weaponize various arenaviruses were reportedly made by the Soviet Union during the Cold War era (14). Many arenaviruses have been classified as biosafety level four (BSL-4) or "maximum containment" (15).

VIROLOGY

Classification

Arenaviruses are grouped serologically, phylogenetically, and geographically into Old World/Lymphocytic Choriomeningitis (i.e., Africa, Europe, Asia, and Oceana) and New World/Tacaribe (i.e., the Americas) complexes (Figure 3). As with other causes of VHF, arenaviruses are generally named after the geographic location of the first recognized case or place of first virus isolation. For the New World arenaviruses, the diseases are often named after the country where they were first detected, with the virus named after a local geographic feature. For example, Junín virus was first isolated from a human in the town of Junín, in northern Argentina, and subsequently designated as the causative virus of Argentine hemorrhagic fever.

Both Old and New World complexes are further divided into major lineages or clades. Genetic diversity within virus species may vary with the host and geographic region. Sequence diversity is generally higher among strains obtained from rodents than from humans, reflective of chronic infection and frequent transmission among the rodents (16, 17).

The Old World complex is grouped into two monophyletic lineages that correlate with monophyletic genera within the rodent family Muridae, subfamily Murinae (see below). The most attention has been paid to characterizing Lassa viruses, which show considerable sequence heterogeneity across West Africa, with 4 recognized lineages—3 in Nigeria and 1 in the area comprising Sierra Leone, Liberia, Guinea, and Ivory Coast (18, 19). There is also considerable genetic heterogeneity within lineages (20). The largest variation is in the L and Z genes, with mean differences of 26% and 20% at the nucleotide and amino acid levels, respectively. The structural genes nucleoprotein (NP) and glycoprotein (GPC) are more conserved, with mean differences of about 20% and 8% at the nucleotide and amino acid levels, respectively (21). Phylogenetic analyses suggest that Nigerian strains are ancestral to those found further west in Africa (19). Migration across the region was accompanied by changes in genome abundance, fatality rates, codon adaptation, and translational efficiency, with the virus evolving to evade immune-determined selection pressures (22).

Field and laboratory data suggest variation in virulence among the 4 lineages and strains of Lassa virus. In laboratory

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TABLE 1 Arenaviruses known or suspected to cause human disease

Virus	Associated human disease	Annual incidence of human disease	Human disease-to-infection ratio	Human-to-human transmissibility	Case fatality
Old World					
Dandenong	Fever with encephalopathy and multiorgan-system failure	Only 3 cases recognized	Unknown	All known cases infected through organ transplantation	100% of 3 known cases
Lassa	Lassa fever	Poor surveillance. Estimated 30,000–50,000	1:5–10	Moderate	20% of hospitalized cases, but mild or asymptomatic infection frequent
Lujo	Lujo fever	Only 5 cases recognized	Unknown	Moderate-to-high	80% of 5 known cases
Lymphocytic choriomeningitis	Nonspecific febrile illness/aseptic meningitis	Estimated 100s to 1,000s but poor surveillance. Incidence declining with improved housing and sanitation.	Unknown but appears to be low	Congenital transmission as well as transmission through organ transplantation	<1%
New World					
Chapare	Chapare HF	Small cluster reported in 2003. None since.	Unknown	Unknown	1 fatality reported among small cluster
Flexal	Nonspecific febrile illness	2 laboratory infections	Unknown	Unknown	None of 2 known cases
Guanarito	Venezuelan HF	<50	1:1.5	Low	30–40%
Junín	Argentine HF	50–100. Incidence declining due to vaccination.	1:1.5	Low	15–30%
Machupo	Bolivian HF	<50	1:1.5	Low	15–30%
Pirital	Nonspecific febrile illness	1 laboratory infection	Unknown	Unknown	One known case was nonfatal
Sabiá	Brazilian HF	1 natural and 2 laboratory infections	1:1.5	Low?	33% of 3 known cases
Tacaribe	Febrile illness with mild CNS symptoms	1 laboratory infection	Unknown	Unknown	Single nonfatal case
Whitewater Arroyo	Pathogenic potential unclear. 3 putative cases with fever and some signs of hemorrhagic fever	Unknown	Unknown	Unknown	Unknown

HF, hemorrhagic fever.

experiments, virulence of Lassa virus strains in guinea pigs roughly correlates with the severity of disease in the humans from whom the viruses were isolated (23). However, strains isolated from pregnant women and infants, in whom disease was often severe, were often completely benign in guinea pigs, suggesting that host factors such as immunosuppression play a role in human disease. Strains of Lassa virus from Nigeria may be more virulent than those from farther West in Africa, but data to support or refute this theory are lacking. The virulence factors of the Lassa virus genome are not known, although for LCMV they are suspected to map to the L segment (24).

The New World complex is classified into 4 distinct lineages, A, B, C, and D, that generally correlate with monophyletic genera of the rodent family Cricetidae (see below). All of the pathogenic New World viruses are in lineage B. Viruses of lineage D appear to be the product of recombination between viruses of lineages A and other arenaviruses, and are thus also known as lineage A/Rec. Lineage A, B, and C viruses are restricted to South America, while lineage D is exclusively North American.

There is also considerable genetic diversity among strains of LCMV, Guanarito, and Mopeia viruses, but no clear relationships between strain and pathogenicity in humans has been sought or recognized. Sequence diversity may also exist for other arenaviruses but the matter has not been extensively studied. Although pathogenic viruses generally cluster phylogenetically, Lujo virus, first identified in 2008, illustrates the limitations in predicting clinical syndromes based on genetic sequence alone (2); Lujo and Lassa viruses cause almost identical VHF syndromes, despite being genetically distinct (up to 38.1% on the nucleotide level)(25). Lujo virus is an outlier between New World and Old World arenaviruses but appears to be closest to the Old World viruses, corresponding to its site of isolation in Zambia.

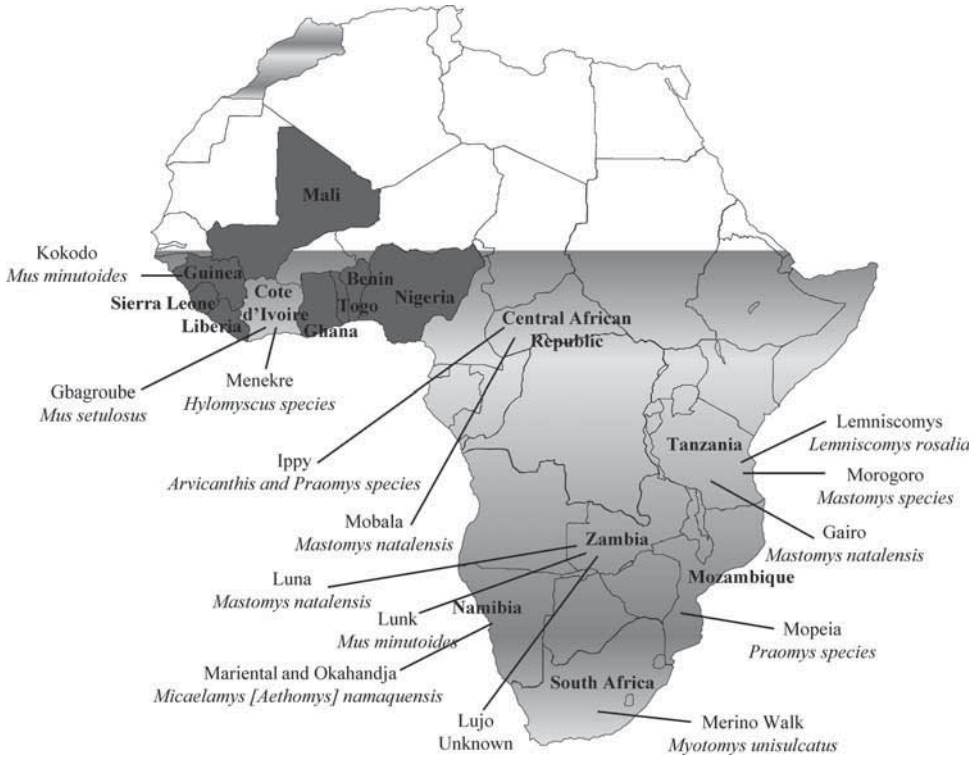

FIGURE 1 Geographic distribution of Old World arenaviruses. The virus name and known or suspected rodent reservoir are listed. Although the virus has been isolated from the animal listed, the status of that animal as the natural reservoir is not established in all cases. Countries where Lassa fever, the major arenaviral disease in Africa, has been definitively shown are depicted in dark gray and the distribution of rodents of the genus *Mastomys* is shown in light gray. Only Lassa, Lujo, lymphocytic choriomeningitis, and Dandenong viruses have been definitively associated with human disease. The distribution of the virus and incidence of associated disease may vary significantly within each country. With the exception of Lassa and lymphocytic choriomeningitis viruses, most Old World arenaviruses have been isolated on single or very few occasions and the precise distribution of the virus beyond the place of first identification is unknown. Not shown on the map: Lymphocytic choriomeningitis viruses (reservoir *Mus musculus*), which has a worldwide distribution, Dandenong virus (unknown reservoir), which is thought to be found in Eastern Europe, and Wenzhou virus (reservoir various rodent species and *Suncus murinus*) found in China.

Virus Composition

Arenaviruses derive their name from the Latin "arena" for "sand," referring to the grainy appearance of infected host cell ribosomes seen in virions on electron microscopy (Figure 4)(26). Surface glycoproteins appear as club-shaped projections or spikes protruding from lipid envelope membrane (26). The virions are pleomorphic, ranging from 60 to 300 nM. Arenaviruses are bisegmented with an ~11 kb genome comprised of two single-stranded RNA segments denoted small (S) and large (L) of 3.4 kb and 7.2 kb, respectively (27, 28) (Figure 5). The S RNA encodes the viral NP and a precursor GPC that is posttranslationally cleaved into GP1 and GP2 by the cellular subtilase SK1-1/S1P. Proteolytic processing of GPC is necessary for arenavirus infectivity. GP1 is an outer membrane protein while GP2 is transmembrane. Both are involved in receptor binding and cell entry. The L RNA encodes the viral polymerase (L protein) and a small zinc-binding protein (Z), which appears to play a regulatory role in virus replication, particle formation, and budding, as well as having a structural function as a matrix protein. The genes on the two RNA segments are separated by an intergenic region that folds into a stable secondary structure.

Replication

Arenavirus genes are oriented in both negative and positive senses on the two RNA segments, a coding strategy called ambisense (Figure 6). Through this mechanism, GPC and NP gene expression are independently regulated. Viral RNA must be transcribed before GPC can be expressed, which may be fundamental to the maintenance of persistent infection in the animal reservoir. Replication and transcription of the genome occur in the cytoplasm and require the association of viral proteins with the viral RNA in the form of ribonucleoprotein (RNP) complexes. The NP and L proteins, together with virus RNA, are the minimal components of the RNP complex and are sufficient for genome replication and transcription. Purified RNPs are competent for RNA synthesis *in vitro* and can initiate virus replication after transfection into cells. Naked RNA is not infectious.

During genome replication, a full-length copy of the genome is synthesized yielding the corresponding antigenomic S and L RNAs. Due to the ambisense coding strategy, both genomic and antigenomic RNA serve as templates for transcription of viral mRNA. The transcripts contain a cap but are not polyadenylated. The viral RNA species that are packaged into virions are defined as the genomic RNAs; however, smaller amounts of antigenomic RNA and Z gene mRNA are also packaged. In addition to viral RNA, ribosomal RNA is present within virions. Virions mature by budding from the plasma membrane.

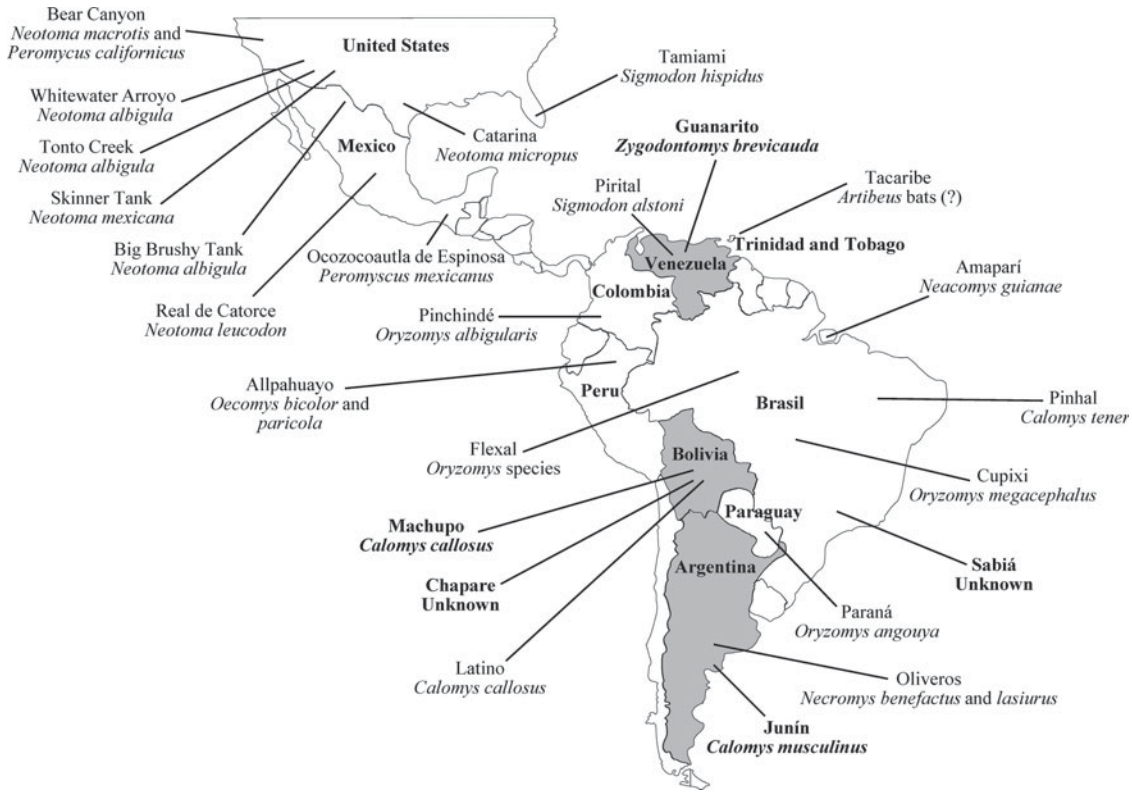

FIGURE 2 Geographic distribution of New World arenaviruses. Above: The virus name and known or suspected rodent reservoir are listed, with viruses associated with natural infection and disease in humans in bold. Although the virus has been isolated from the animal listed, the status of that animal as the natural reservoir is not established in all cases. Countries with arenaviruses definitively associated with human disease are shaded gray. The distribution of the virus and incidence of associated disease may vary significantly within each country. Many of the New World arenaviruses have been isolated on single or very few occasions and the precise distribution of the virus beyond the place of first identification is unknown. On next page: Closeup of administrative regions in which Venezuelan, Bolivian, Chapare, and Argentine hemorrhagic fever have been recognized. The distribution of the viruses and incidence of disease may vary significantly within the region.

Receptors

Arenaviruses enter cells by attachment of the GP1 to one or more cellular receptors (29); α-dystroglycan, a protein found ubiquitously on primate and rodent cells, is a principal receptor for Old World viruses and pathogenic viruses of the New World clade C, while human transferrin receptor 1 protein is a receptor for the New World clade B arenaviruses (30). The transmembrane proteins Axl, Tyro3, dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN), and liver and lymph node sinusoidal endothelial calcium-dependent lectin (LSECtin) may also serve as Lassa virus receptors independent of α-dystroglycan (31). GP2 is thought to mediate fusion of the viral envelope with the cellular membrane, and thus entry of the virus into the host cell. Lassa virus and other nonpathogenic arenaviruses bind α-dystroglycan with equal affinity. Orthologs of transferrin receptor 1 protein appear to be the major receptors in the respective rodent host of each New World arenavirus and likely dictate species specificity (32).

Animal Reservoirs and Host Range

Arenaviruses are generally maintained in nature by chronic infection in rodents of the superfamily Muroidea (Figure 4) (1). Transmission may be vertical (dam-to-progeny) or horizontal, depending on the specific arenavirus. Infected animals may chronically shed virus in urine, feces, and saliva. Old World arenaviruses are maintained in rodents of the family Muridae, subfamily Murinae, and New World arenaviruses in the family Cricetidae, subfamilies Sigmodontinae and Neotominae. There is a tight host-virus species pairing, thought to be the result of long-term rodent-virus coevolution (1)(Table 2). Occasional findings of a given arenavirus in species other than its recognized rodent host are usually considered to result from spillover infection (i.e., incidental transient infection of a nonreservoir host). These incidental animal hosts do not play a role in long-term virus maintenance, but may nevertheless still pass virus on to humans who are exposed during the period of transient infection in the rodent.

The endemic area of each arenaviral disease is restricted to the geographic distribution of its rodent reservoir. Rodent populations are usually not uniformly infected; rather, the distribution of the virus and disease is usually restricted to a small portion of the overall host range. The reasons for this are unclear, but may relate to evolutionary bottlenecks in dispersal of the virus, rodent reservoir, or both. Landscape features are the most likely barriers to host migration. Both rodent abundance and prevalence of infection are highly

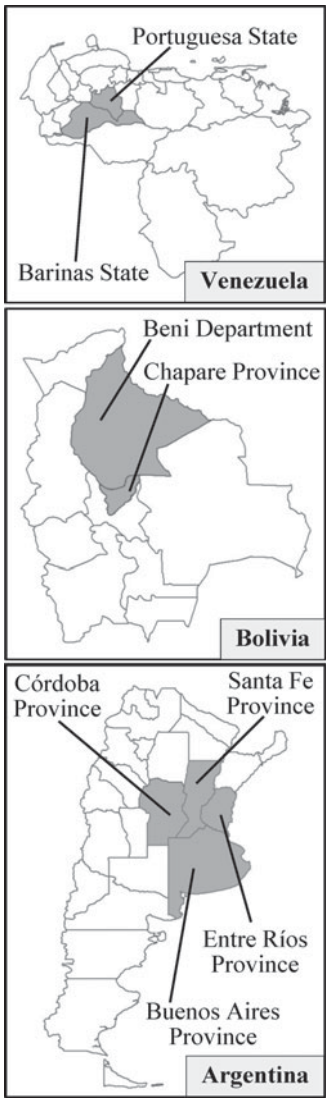

FIGURE 2 (Continued)

variable over space and time, with the prevalence of virus infection in reservoir populations likely linked to population density. Humans are dead-end hosts who play no role in the natural maintenance of arenaviruses. There are generally few human cases relative to the frequency of infected rodents. Incidence of human infection may vary with changes in rodent abundance that relate to both climatic and seasonal weather changes and human-induced habitat alterations. Detailed discussions of the epizoology of arenaviruses and their animal reservoirs have been reviewed elsewhere (1, 33).

Growth in Cell Culture

Arenaviruses grow well in most mammalian cell lines, including endothelial cells, and infect most mammals. Vero cells are most commonly used, in which most cells are productively infected in 4 to 9 days with peak infectivity titers approaching 10^8/ml. Carrier cultures are readily established with cyclic production of infectious virus. Although rounding and detachment of affected cells and subsequent cell death are occasionally seen, arenaviruses generally produce little or no cytopathic effect, so all cultures should be harvested and examined by immunofluorescent antibody (IFA) assay using virus-specific antiserum (34). Infected cells from these cultures may contain large quantities of N protein but minimal GPC. There is evidence for the presence of defective interfering particles that can suppress replication both in cell culture and animals but the significance of this observation relative to natural infection is unknown (35).

Animal Models

Models for arenavirus propagation and disease have been developed for suckling mice, hamsters, guinea pigs, and monkeys. In addition to being a human pathogen in its own right, LCMV has played a major role in elucidating our concepts of viral immunopathology and of T-cell function through experiments with laboratory strains of virus and inbred mice (36).

Inactivation by Physical and Chemical Agents

The arenavirus lipid envelope is easily disrupted, limiting their viability outside a living host.

Arenaviruses can be inactivated by heating to 60°C for 1 hour, use of disinfectants containing phenolic compounds, hypochlorite, quaternary amines, acidic or basic pH, ultraviolet light (surface disinfection only), gamma irradiation and surfactant nanoemulsions, and various proprietary detergent-containing lysis buffers (37–51). Some of these inactivation techniques, such as heat and gamma irradiation, preserve the arenavirus proteins for serologic testing. Toxicity and corrosion may be concerns with some of these compounds depending upon the frequency of use and concentrations.

The transmission dynamics and viability of arenaviruses after shedding into the environment are not well characterized. However, there is generally little concern required regarding arenaviruses seeping into groundwater or posing any long-term risk through casual exposures in the general environment, where harsh thermal and pH conditions would likely readily inactivate them. When shed naturally in animal excreta or human body fluids, which would then usually dry, infectivity appears to last from a few hours to days, varying with the specific virus and environmental conditions such as temperature and light (52–54). However, other nonarenavirus causing VHF have been isolated from samples kept for weeks at ambient temperatures if stored hydrated in a biological buffer, such as blood or serum. This may hold true for arenaviruses as well. The viruses are also stable when frozen or freeze-dried (55).

EPIDEMIOLOGY

Arenavirus infections are noted in both sexes and all age groups. The geographic distribution and impact of arenaviral infections vary widely by particular agent (Table 1). The combination of the remote, geographically restricted endemic areas (often in resource-poor countries), nonspecific clinical presentation (56, 57), lack of availability of reagents and laboratories for diagnostic confirmation (58), and, in some areas, logistical impediments presented by civil unrest (59) and unstable governments (60) make surveillance for arenavirus infection, and thus precise estimates of incidence, difficult (61). One exception is Argentine hemorrhagic fever, for which intensive surveillance with supporting laboratory diagnosis is applied in its circumscribed endemic area, resulting in precise incidence estimates—usually less than 100 cases per year. Lassa virus is the most common arenavirus infection, with upwards of 50,000 cases and an estimated 10,000 deaths annually. LCMV may cause up to several thousand infections yearly, whereas much smaller

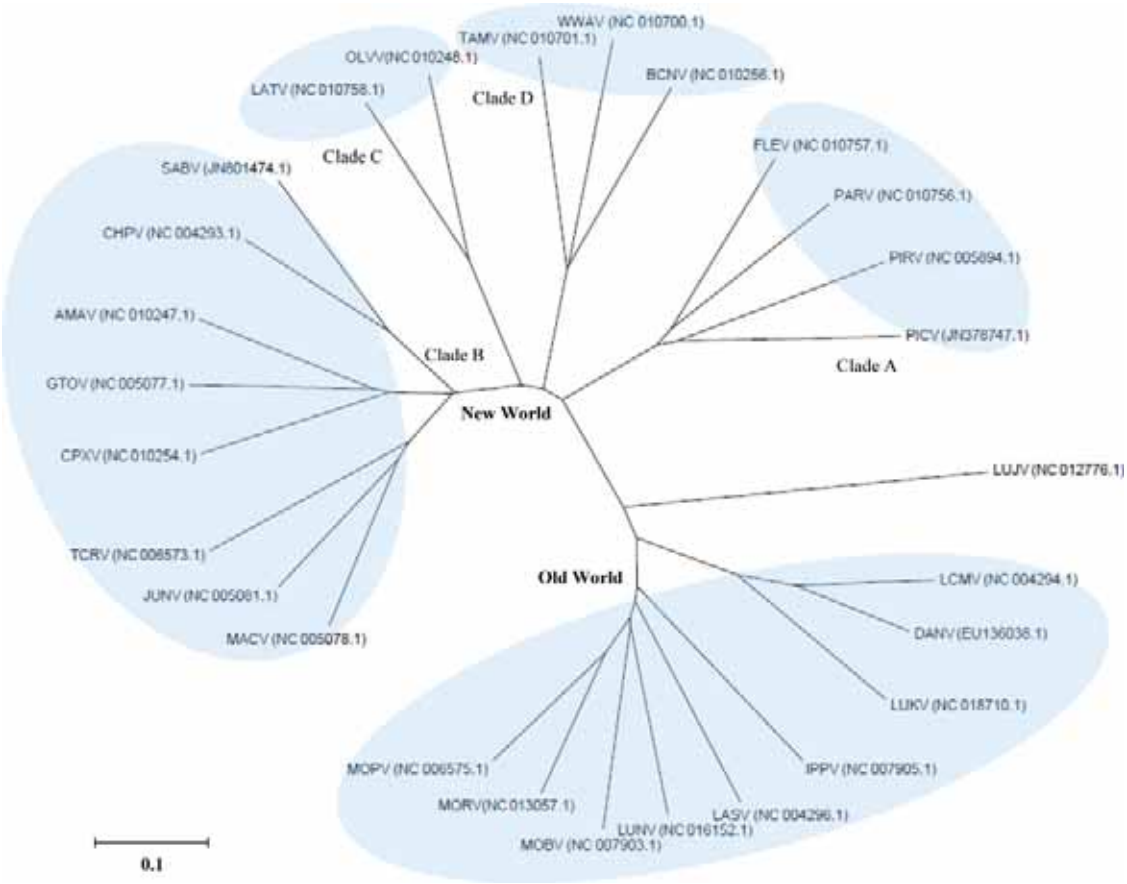

FIGURE 3 Phylogenetic relationships of arenaviruses inferred based on full S segment nucleotide sequences. Phylogenies were reconstructed by neighbor-joining analysis applying the JUKES-Cantor model (1,000 replicates). The A, B, C, and D clades of the New World and Old World arenaviruses are delineated. Lujo virus (LUJV) falls between the two groups but appears to be closest to the Old World viruses, thus corresponding to its site of isolation in Zambia. The scale bar indicates substitutions per site. Genbank accession numbers are shown after each virus. Similar relationships are demonstrated based on L segment analysis (not shown). Abbreviations: AMAV, Ampari virus; BCNV, Bear Canyon virus; CHPV, Chapare virus; CPXV, Cupixi virus; DANV, Dandenong virus; FLEV, Flexal virus; GTOV, Guanarito virus; IPPV, Ippy virus; JUNV, Junín virus; LASV, Lassa virus; LATV, Latino virus; LCMV, Lymphocytic choriomeningitis virus; LUJV, Lujo virus; LUKV, Lunk virus; LUNV, Luna virus; MACV, Machupo virus; MOBV, Mobala virus; MOPV, Mopeia virus; MORV, Morogoro virus; OLVV, Oliveros virus; PARV, Paraná virus; PICV, Pichinde virus; PIRV, Pirital virus; SABV, Sabiá virus; TCRV, Tacaribe; TAMV, Tamiami virus; WWAV, Whitewater Arroyo virus. Viruses not shown due to incomplete or unavailable sequence data: Allpahuayo, Big Brushy Tank, California Academy of Sciences, Catarina, Collierville, Gairo, Gbagroube, Golden Gate, Kodoko, Lemniscomys, Mariental, Menekre, Merino Walk, Ocozocoautla de Espinosa, Okahandja, Pinhal, Real de Catorce, Skinner Tank, Tonto Creek, and Wenzhou.

numbers of human cases have been recognized for the other arenaviruses.

While New World arenavirus infection usually results in disease that can ultimately be recognized as VHF, the majority of Old World arenavirus infections, at least for LCMV and Lassa virus, are thought to be asymptomatic or produce milder nonspecific febrile illness (62). However, antibody reversion (i.e., loss of antibody after infection) after Lassa virus infection and the possibility of cross-reacting antibodies from previous infection with other nonpathogenic arenaviruses in West Africa, such as Kodoko, Gbagroube, or Menekre viruses (Figure 1), may confound interpretation of laboratory findings, potentially resulting in misclassification between true first Lassa virus infections and reinfection, which is associated with milder disease. Studies of the rate of asymptomatic transmission as well as reinfection bear repeating with newer more sensitive and specific diagnostic modalities (63, 64).

Because arenaviruses are maintained in wild animals, factors in disease emergence and increased incidence of human disease logically often relate to anthropogenic disturbance of natural habitats, such as conversion of forest to cultivated fields, resulting in loss of biodiversity and selection for opportunistic rodent species that are frequently hosts for zoonotic pathogens (Table 2)(65). Conversion to agriculture also prompts incursion of reservoir rodents from the surrounding bush seeking food in croplands, with resultant increased human exposure to rodents, usually with recognizable seasonal peaks (66, 67). Some reservoir species may also enter towns and homes, further increasing risk to humans.

Rodent-to-Human Transmission

With the exception of the LCMV reservoir, all arenavirus reservoirs occupy almost exclusively sylvatic rural habitats. Consequently, primary arenavirus infections in humans are seen almost exclusively in rural and often remote settings.

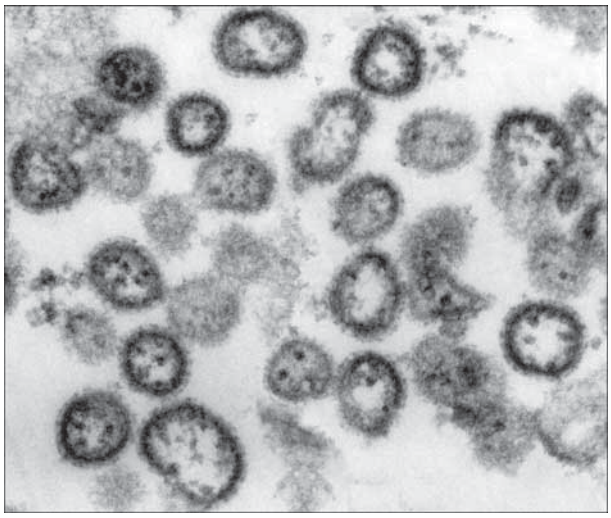

FIGURE 4 Electron micrograph of Lassa virus. The typical sandy appearance of arenaviruses from the internal ribosomes is evident, as well as the surface glycoprotein spikes. Magnification approximately ×55,000. Micrograph courtesy of F. A. Murphy, University of Texas Medical Branch, Galveston, Texas.

Virus transmission to humans is believed to occur via exposure to rodent excreta, either from direct inoculation on to the mucous membranes or broken skin, or from inhalation of aerosols produced when rodents urinate (1, 62, 68, 69). The relative frequency of these modes of transmission is unknown. Although the infectious dose for arenaviruses is thought to be low, rodent-to-human transmission appears to be inefficient, occurring infrequently even where infected rodents are common (70). Transmission by aerosolized rodent urine or virus-contaminated dust particles is often mentioned in the scientific literature, but there are few data to support or refute its occurrence (71). Although household clusters of VHF due to arenaviruses occasionally occur, single cases are much more common, suggesting that aerosol transmission to humans is not common, since it would logically often simultaneously infect multiple people in proximity to the aerosol source (72). Secondary aerosol generation,

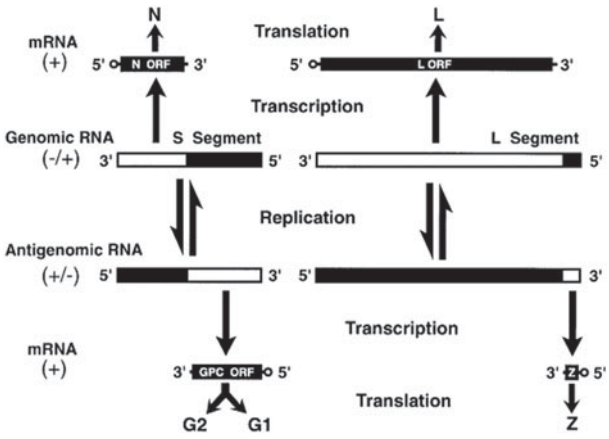

FIGURE 5 Replication strategy of arenaviruses. See text for details. (Prepared by A. Sanchez.)

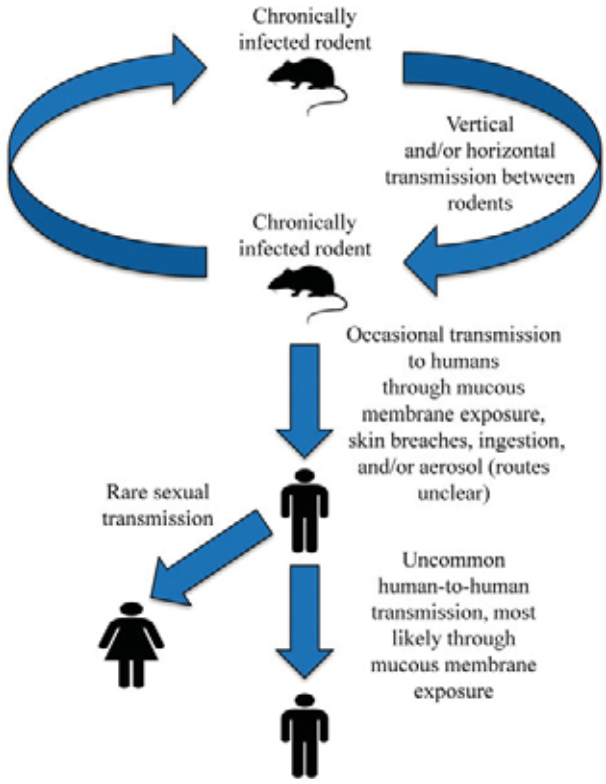

FIGURE 6 Transmission cycle of arenaviruses illustrating chronic infection in rodents. Transmission between rodents may be vertical or horizontal depending upon the specific arenavirus. Humans are incidental hosts who play no role in virus maintenance in nature. (Adapted from reference 13 with permission of the publisher.)

such as what might be produced through sweeping an area contaminated by rodent urine, is inefficient and is thus a less likely mechanism of infection. However, infectious and moderately stable aerosols of Lassa, LCMV, and Junín viruses have been artificially produced in the laboratory, and a laboratory infection with Sabiá virus resulted from a centrifuge accident (73) so the possibility of aerosol infection is not zero (74, 75). Regardless of their role in natural infection, the artificial production of infectious aerosols has obvious implications for the potential use of arenaviruses as bioweapons (15, 76).

Experiments in mice and monkeys show that arenavirus infection may also occur by the oral route, perhaps through a gastric portal (77, 78). In a monkey model using LCMV, hemorrhagic fever was produced with intravenous (IV) but not intragastric inoculation (78). Lassa virus has been contracted when rodents are trapped and prepared for consumption, a common practice in some parts of West Africa, although it is usually impossible to determine whether infection resulted from exposure during preparation or consumption (79). LCMV has been identified in wild rodent feces by reverse transcriptase polymerase chain reaction (RT-PCR) (80). Since arenaviruses are easily inactivated by heating, eating cooked rodent meat should pose no danger (1). Various arenaviruses have been found in rodent saliva, although reports of human infection from rodent bites are rare (81, 82). Although the primary reservoir for LCMV is the common house mouse (*Mus musculus*), spillover

TABLE 2 Natural reservoirs of the arenaviruses

Virus	Primary reservoir-*species* (common name)	Mode of virus maintenance in animals	Typical habitat	Known geographic distribution of infected rodents	Seasonal trends and risk factors for human exposure
Dandenong	Unknown	Unknown	Unknown	Balkan Peninsula?	Unknown. All known cases infected through organ transplantation in Australia
Lassa	*Mastomys natalensis* (natal mastomys or multimammate rat)	Vertical	Peridomestic and surrounding cultivated fields and grasslands. Rural areas only	West Africa, especially Nigeria, Sierra Leone, Liberia, and Guinea	Peak transmission in dry season (November–April). Rodent consumption, poor quality housing, open food storage and closed shutters during the day, favoring prolonged foraging
Lujo	Unknown	Unknown	Unknown	Zambia?	Unknown
Lymphocytic choriomeningitis	*Mus musculus* (house mouse)	Vertical	Peridomestic. Rural and urban areas. Most infections inside homes	Worldwide	Peak transmission in fall and winter in northern temperate zones due to seasonal invasion of homes by house mice. Also risk among rural dwellers and low-income groups Infections documented from infected laboratory mice and pet mice and hamsters
Chapare	Unknown	Unknown	Unknown	Chapare region of Cochabamba, Bolivia	Unknown
Flexal	Unknown	Unknown	Unknown	Unknown	Only known cases through laboratory infection
Guanarito	*Zygodontomys brevicauda* (short-tailed zygodont mouse or cane mouse)	Split horizontal/vertical response	Crop fields, borders, and roadside habitats in rural areas. Rarely peridomestic	Plains of western Venezuela, especially Portuguesa and Barinas States	Peak incidence during peak agricultural activity (November–January). Agricultural workers highest risk. Often 4–5 year cyclical pattern

Junín	*Calomys musculinus* (drylands vesper mouse or corn mouse)	Split horizontal/vertical response	Stable linear border habitats (roadsides, fence lines, and railroad rights of way) in rural areas. Movement into mature and postharvest crop fields in summer and early fall	Central and northwestern Argentina	Peak incidence during peak agriculture activity (March–June). Agricultural workers highest risk
Machupo	*Calomys callosus*-Beni Department clade (big laucha or large vesper mouse)	Split horizontal/vertical response	Peridomestic and disturbed habitats where forest and grasslands meet. Seeks higher ground with seasonal inundations. Rural areas	El Beni Department of Bolivia	Peak incidence during peak agricultural activity (June–August). Agricultural workers at highest risk but family and community clusters occasionally noted
Pirital	*Sigmodon alstoni* (Alston's cotton rat)	Unknown	Crop fields, borders, and roadside habitats in rural areas. Rarely peridomestic	Plains of northwestern Venezuela	Only known cases through laboratory infection
Sabiá	Unknown	Unknown	Unknown	Rural area near Sao Paulo, Brazil?	Only known case outside of laboratory infections occurred during dry season (January)
Tacaribe	*Artibeus* bat*	Unknown	Unknown	Trinidad	Only known cases through laboratory infection
Whitewater Arroyo	Related viruses found in various species of *Neotoma* genus, including *N. micropus* (southern plains woodrat), *N. Mexicana* (Mexican woodrat), (*N. stephensi* (Stephen's woodrat), and *N. cinerea* (bushy-tailed woodrat)	Unknown	Rural areas	Southwestern United States	Unknown

* Tacaribe virus has been isolated from mammals only once, from bats from the genus *Artibeus*, but the definitive role of this animal as the natural reservoir is uncertain. The virus has also been isolated from ticks in the state of Florida in the United States (202).

infection can occur in a variety of pet animals, such as Syrian hamsters (*Mesocricetus auratus*) and guinea pigs (Cavia porcellus), and laboratory mice, and have resulted in human infection (83).

Human-to-Human Transmission

With the exception of LCMV, and perhaps Dandenong virus, arenaviruses can be transmitted between sick humans through direct contact with infected blood or bodily fluids. However, contrary to popular concept, secondary attack rates are generally low, probably of the order of 5% or less as long as strict barrier nursing practices are observed (Table 1). Tertiary transmission is unusual. Large outbreaks are almost always fueled by nosocomial transmission, usually in resource-poor regions where sound infection control and barrier nursing practices may not be maintained (84–87). Nosocomial outbreaks of Lassa fever have been associated with reuse of nonsterilized needles and use of contaminated multiuse antibiotic vials (84).

Viremia and infectivity of persons infected with arenaviruses generally parallel the clinical state, with highest infectivity late in the course of severe disease, especially when bleeding is present. Data on the precise modes of human-to-human transmission are lacking, but infection presumably results from oral or mucous membrane exposure, most often in the context of providing care to a sick family member (community) or patient (nosocomial transmission). Although aerosol spread of Lassa virus was speculated in the first recognized outbreaks in Nigeria, extensive field experience since then has not suggested aerosol transmission of arenaviruses between humans in natural settings (86). Funeral rituals that entail the touching of the corpse prior to burial may also result in transmission of hemorrhagic fever viruses, although this has not been specifically recognized with arenaviruses (88). Although a few anecdotal reports exist of transmission during convalescence disease (62, 89), presumably from virus persistence in the semen as noted in some other VHFs, there are no reports of virus isolation from the semen. However, intermittent shedding of Lassa virus in the urine has been documented up to 67 days after the onset of illness (90, 91). Transmission during the incubation period has not been noted.

New World arenaviruses appear to be less transmissible between humans than their Old World counterparts, although human-to-human transmission of Machupo virus has been reported in both community and nosocomial settings (92, 93). Only one family cluster of Argentine hemorrhagic fever is described, with the index case presenting with atypical skin lesions that may have facilitated transmission. Person-to-person transmission or nosocomial infection has not been observed with Guanarito virus despite the fact that patients with Venezuelan hemorrhagic fever are usually admitted to open wards with minimal isolation precautions. It is unknown whether the perceived differences in transmissibility between the arenaviruses reflect true biological properties or varied cultural and infection control practices in the endemic areas of each virus.

Contrary to popular misconceptions, the risk of imported arenavirus infections initiating outbreaks in industrialized countries, where barrier nursing techniques are usually maintained, is generally low. Because of the considerable travel between the United Kingdom and their former West African colonies, which also often happen to be the hyperendemic areas, Lassa fever is the most frequently exported arenavirus infection. Foreign military personnel, peacekeepers, aid workers, and tourists in rural settings are occasionally infected, sometimes importing Lassa virus back to their countries of origin, most commonly the United Kingdom and Europe, but occasionally as far away as Japan (94). In over 25 imported cases of Lassa fever reported since 1969 with at least 1,500 cumulative identified contacts, only a single putative and asymptomatic instance of secondary transmission has been noted (95, 96). Although 3 secondary cases and 1 tertiary case occurred in South Africa after the arrival of a traveler from Zambia infected with Lujo virus, transmission occurred in settings in which proper infection control practices may not have been maintained (2, 97).

Small clusters of severe neurologic disease and organ failure have been reported in the United States among persons who were transplanted solid organs from persons unknowingly recently infected with LCMV (98, 99). A similar transplant-related cluster of 3 fatal cases occurred in Australia in 2009, leading to the discovery of the LCMV variant Dandenong virus (100). The donor died of cerebral hemorrhage 10 days after returning to Australia from a 3-month visit to the Balkan Peninsula, where he had traveled in rural areas. None of the donors in these outbreaks had a recent history of acute febrile disease and no virus could be identified in them, although the presence of IgG and IgM antibodies in the Australian donor confirmed recent infection. One donor in the United States had been exposed to a pet hamster from which LCMV was isolated and corresponded to the virus detected in the transplant recipients. With the exception of congenital transmission and through transplanted organs, there is no human-to-human transmission of LCMV.

PATHOGENESIS

Incubation Period

The incubation period for arenavirus infection is typically between 8 and 13 days, with a range of 3 to 21 days. Direct percutaneous inoculations, such as needlesticks, are thought to be associated with shorter incubation periods.

Pathology

A recurring question with the arenavirus infections is how the virus causes disease. The histologic lesions observed do not usually provide an obvious cause of death; overt cell damage by arenaviruses is minimal or modest. Rather, arenaviruses are thought to alter cell function and through direct infection or indirectly through immunopathologic mechanisms. Extensive macrophage and lymphoreticular infection are thought to be the basis for local and systemic release of physiologically active soluble mediators leading to many of the abnormalities observed (101). Findings at necropsy are often composed of petechial and mucosal hemorrhages, vascular dilatation and congestion, and small foci of necrosis in organs such as the liver, adrenals, and kidneys, with a mild or absent inflammatory response (155). The pathogenesis of LCMV and Dandenong viruses causing CNS disease appears to be distinct from that of the arenaviruses that cause VHF.

Patterns of Virus Replication and Immune Responses

Central Nervous System Disease

Laboratory studies show arenavirus tropism for Schwann cells (though not neurons), which abundantly express α-dystroglycan (102). Immune cell activation, particularly CD8+ lymphocytes, is thought to be the fundamental process in the pathogenesis of LCMV, although without the same

effects on endothelial cell function and hemostasis as in VHF (see below) (13). CNS involvement typically occurs a week or two after disease onset, after the virus has cleared from the blood, and is thus thought to be immune mediated, although virus can still be recovered from the CSF at this time.

Arenaviral Hemorrhagic Fever

As with all VHFs, microvascular instability and impaired hemostasis are the hallmarks of arenaviral hemorrhagic fever. Unchecked viremia appears to be central to the pathogenesis (103, 104). While both Old and New World viruses cause disruption of the vascular endothelium, the immune responses of these two groups appear to be distinct.

Abnormal platelet aggregation has been noted in Lassa fever and is thought to be the primary functional defect resulting in bleeding when it occurs (105). Old World viruses generally result in suppression of the innate immune response (106–108), while New World viruses elicit a strong inflammatory response consistent with cytokine storm (109, 110). Tumor necrosis factor alpha and alpha/beta interferon are among the candidates for such mediators; both have been found at high concentrations in the blood that correlate with mortality in patients with Argentine hemorrhagic fever (111). In animal models, leukotrienes, platelet-activating factor, and endorphins appear to contribute (74). Apoptosis has also been reported from experiments with New World viruses in cell and animal models (112). Immune complexes, disseminated intravascular coagulation, and complement activation by other means appear to have no important role in arenaviral hemorrhagic fever.

Viremia is usually present at patient presentation (presumably starting with disease onset, although patients are rarely available for testing at this time), peaking between days 4 and 9 and clearing within 2 to 3 weeks in survivors. Cell mediated immunity is thought to be primarily responsible for clearance of Old World arenavirus infection, while the humoral arm is important in disease caused by New World viruses (113–115). Antibody titers are significantly lower in fatal cases relative to survivors (63, 103, 116). Neutralizing antibodies may be produced in Old World arenavirus infection, but usually months after recovery and often at a low titer. The continued increase in antibody titer to Lassa virus months after infection suggests a sustained B-cell response that might be attributable to low-level virus persistence in immunologically protected sites; in vaccine experiments in monkeys, replication-competent virus was cleared within 14 days after Lassa virus challenge, but detection of RNA up to 112 days suggested low-level viral persistence or the presence of defective interfering particles (117).

A long-standing mystery of arenavirus infection, especially Lassa fever, is the apparent extreme range of clinical severity (28). The reasons for this variation are unknown, but may relate to heterogeneity in the virulence of infecting Lassa virus strains, route and dose of inoculation, genetic predisposition, underlying coinfections or premorbid conditions (e.g., malaria, malnutrition, or diabetes), or misclassification of reinfection as new infection due to waning of antibody. Unfortunately, since most studies in nonhuman primates use viral challenge doses designed to produce uniformly fatal disease in order to evaluate the efficacy of therapeutics and vaccines, they shed little light on any clinical spectrum related to inoculum size. Route of exposure appears to be one important variable; in a monkey model of Lassa fever using the WE strain of LCMV, IV inoculation resulted in fatal VHF while monkeys inoculated via the gastric route mostly had an attenuated infection with no disease (78, 113). Human genes encoding "like glycosyltransferase" (*LARGE*), dystrophin (*DMD*), and IL-21 have apparently undergone positive selection in populations in endemic areas for Lassa fever in Nigeria, suggesting a protective effect (118, 119).

CLINICAL MANIFESTATIONS

Central Nervous System Disease

Despite the name, a minority of patients infected with LCMV develop meningitis or CNS disease. Rather, most LCMV infections are asymptomatic or result in a nonspecific febrile illness (120). After 7 to 14 days of nonspecific illness (fever, headache, malaise), and often with a brief period of deffervescence, CNS manifestations may ensue in a minority of patients. These encompass a spectrum ranging from aseptic meningitis (the most common) with headache, stiff neck, and photophobia to fulminant encephalitis with cranial nerve palsies, abnormal reflexes, focal seizures, polyneuritis, flaccid paralysis, and papilledema. CNS symptoms can also occur without recognized febrile illness. Rarer manifestations and complications of LCMV infection include hydrocephalus, transverse myelitis, Guillain-Barré syndrome, hearing loss, arthritis, parotitis, orchitis, myocarditis, mucosal bleeding, and pneumonia. Congenital infection has been associated with spontaneous abortion in early pregnancy and, when occurring later in pregnancy, a variety of neurological deficits, including psychomotor retardation, microcephaly and macrocephaly, hydrocephalus, chorioretinitis with visual loss, and seizures. LCMV and Dandenong virus infection in immunosuppressed organ transplant recipients has resulted in a fatal syndrome with graft dysfunction, CNS symptoms, and multiorgan system involvement, in some cases resembling VHF, within three weeks after transplantation, with case-fatality approaching 90% (98).

Arenaviral Hemorrhagic Fever

Disease severity generally correlates directly with the level of viremia, and thus infectivity. Although there are differences in the pathogenesis and clinical manifestations produced by the various arenaviruses, they are usually too subtle to allow for distinction on clinical grounds, at least in the early stages of disease. Nevertheless, some notable distinctions can be made between the syndromes caused by Old World and New World viruses. The disease caused by the various New World arenaviruses is usually referred to simply as "South American hemorrhagic fever."

The spectrum of disease due to arenavirus infection ranges from mild to shock, multiorgan system failure, and death. Most patients present with nonspecific signs and symptoms difficult to distinguish from many other more common febrile illnesses. The incubation period is usually about 1 week (range 3 to 21 days). Illness typically begins with the gradual onset of fever and constitutional symptoms, including general malaise, anorexia, headache, chest or retrosternal pain, sore throat, myalgia, arthralgia, lumbosacral pain, and dizziness (56, 57). The pharynx may be erythematous or even exudative in Lassa fever, a finding that has at times led to misdiagnosis of streptococcal pharyngitis (56). Gastrointestinal signs and symptoms occur early in the course of disease and may include nausea, vomiting, epigastric and abdominal pain and tenderness, and diarrhea. Lassa fever has sometimes been mistaken for acute appendicitis or other abdominal emergencies. A morbilliform, maculopapular, or petechial skin rash is very frequent in South American hemorrhagic fevers (Figure 7A). Although

FIGURE 7 Clinical manifestations of arenaviral hemorrhagic fever. (A) Petechial rash in Argentine hemorrhagic fever. (B) Maculopapular rash in Lujo hemorrhagic fever (photo by T. H. Dinh). (C) Conjunctival injection in Lassa fever (photo by Donald Grant). (D) Facial swelling and mild gum bleeding in Lassa fever (photo by Donald Grant). (E) Gum bleeding in Argentine hemorrhagic fever. Photos used with permission from Blumberg L, Enria D, and Bausch DG. Viral Haemorrhagic Fevers. In J Farrar et al. (ed): *Manson's Tropical Diseases*, 23rd Edition, Elsevier Publishing, 2013.

rash almost always occurs in fair-skinned persons with Lassa and Lujo fever, it is rarely recognized in native black Africans (Figure 7B). The reasons for this observation are unknown, but prior infection with partial immunity and genetic differences have been postulated. Conjunctival injection or hemorrhage (Figure 7C) is frequent but is not accompanied by itching, discharge, or rhinitis. A dry cough, sometimes accompanied by a few scattered rales on auscultation may be noted, but prominent pulmonary symptoms are uncommon early in the course of the disease. Jaundice is not typical and should suggest another diagnosis.

In severe cases, patients progress to vascular instability, which may be manifested by subconjunctival hemorrhage, facial flushing, edema, bleeding, hypotension, shock, and proteinuria. Swelling in the face and neck and bleeding are particularly specific but not sensitive signs in Lassa and Lujo fevers—seen in less than 20% of cases (56) (Figure 7D). Despite the term "VHF," clinically discernible hemorrhage is not always seen, being less frequent in disease produced by Old World than New World arenaviruses, and never in the first few days of illness (56, 57). Hematemesis, melena, hematochezia, metrorrhagia, petechiae, epistaxis, and bleeding from the gums (Figures 7D and 7E) and venipuncture sites may develop, but hemoptysis and hematuria are infrequent. Significant internal bleeding from the gastrointestinal tract may occur even in the absence of external hemorrhage.

Neurological complications are more common in the South American hemorrhagic fevers and include disorientation, tremor, ataxia, seizures, and coma, particularly in the late stages, and usually portend a fatal outcome (121). The cellular and chemistry profile in CSF is often normal. Lassa virus can be isolated from the CSF of some, but not all, patients with neurological manifestations, without apparent correlation between disease severity and detection of virus (103, 122). In one unusual case, Lassa virus was isolated from the CSF, but not the blood, of a patient with encephalopathy after the febrile stage of disease—a finding consistent with virus persistence in the immunologically protected CNS (123).

Pregnant women with arenaviral hemorrhagic fever often present with spontaneous abortion and vaginal bleeding, with maternal and fetal mortality rates approaching 100% in the third trimester (124). Anasarca has been described in a single report of four children with Lassa fever (termed the "swollen baby syndrome") but may have been related to aggressive rehydration (125). One instance of polyserositis with pleural and pericardial effusions and ascites 6 months after infection was reported (126). Lassa virus could not be recovered from the effusion fluid, but lymphocytes and high levels of antibody were noted, suggesting an immune-mediated mechanism. Various clinical manifestations of arenaviral hemorrhagic fever are shown in Figure 6.

Typical clinical laboratory findings in arenaviral hemorrhagic fever include early leukopenia and lymphocytopenia, sometimes with atypical lymphocytes, followed later by leukocytosis with a left shift; mild-to-moderate thrombocytopenia, hemoconcentration; elevated aspartate aminotransferase (AST), alanine aminotransferase (ALT), and amylase; electrolyte perturbations; and proteinuria (103, 127). Unlike classic viral hepatitides, but similar to filoviral disease, the AST is typically much higher than the ALT in arenaviral hemorrhagic fever, suggesting that its source is not exclusively the liver. Since a broad range of tissues can release AST, it should be considered a marker of systemic organ damage (103). Radiographic and electrocardiographic findings are generally nonspecific and correlate with the physical examination (128, 129).

Prognosis

Death in fatal cases of arenaviral hemorrhagic fever usually occurs within 2 to 3 weeks after the onset of disease. Case fatality proportions vary according to the specific infecting

virus (Table 1). Hospital mortality data generally represent an overestimate considering the mild and asymptomatic infections that are thought to frequently occur in the community with some arenaviral hemorrhagic fevers, especially Lassa fever (62). Common indicators of a poor prognosis include shock, bleeding, neurological manifestations, viremia $>10^8$ $TCID_{50}$/ml (or viral RNA copy number or enzyme-linked immunosorbent assay (ELISA) antigen as surrogates), AST >150 IU/l, and pregnancy, especially during the third trimester when maternal and fetal mortality approach 100% (56, 130).

Convalescence and Sequelae

Convalescence from arenaviral hemorrhagic fever may be prolonged, with persistent myalgia, arthralgia, anorexia, weight loss, and alopecia up to a year after infection. Cerebellar ataxia has also been occasionally reported (131). The psychological effects of the disease may also be significant and are often overlooked, with some patients experiencing depression or posttraumatic stress, as well as social stigmatization.

Sensorineural deafness is a well-recognized sequela specific to Lassa fever. It is reported to occur in as many as 25% of cases, although this seems like a significant overestimate from experience in Sierra Leone and Guinea over the last 20 years (132). Deafness typically presents during convalescence and is not associated with the severity of the acute illness or level of viremia, suggesting an immune-mediated pathogenesis (132). It may be uni- or bilateral, and is permanent in approximately two-thirds of cases. Auditory patterns resemble idiopathic nerve deafness (133).

Differential Diagnosis

Difficulties in diagnosing arenavirus infection, both clinically and in the laboratory, pose a major impediment to surveillance and control. Even if more clearly recognized VHF or neurologic syndromes eventually develop, most patients present with a nonspecific febrile syndrome difficult to distinguish from many other diseases that are usually more common in the area. The differential diagnosis of arenaviral hemorrhagic fever includes a broad array of febrile illnesses, including malaria, typhoid fever, leptospirosis, bacterial septicemia, rickettsial infections, dengue fever, and other VHF syndromes, depending upon the specific geographic region and patient history of exposures (127).

The classic presentation of LCMV infection is aseptic meningitis, particularly if an initial prodromal period of fever, perhaps with a remission, occurs before the CNS phase. The differential diagnosis includes the arboviral meningitides, herpes encephalitis and, for congenital manifestations, the classic TORCH organisms (*t*oxoplasma, *r*ubella, *c*ytomegalovirus, and *h*erpes virus). The presence of thrombocytopenia and leukopenia should enhance suspicion of arenavirus infection.

A diagnosis of arenavirus infection should be considered in patients with a clinically compatible syndrome who, within 3 weeks prior to disease onset, (i) lived in or travelled to an endemic area (Figures 1 and 2), (ii) had potential direct contact with blood or bodily fluids of a person with arenaviral hemorrhagic fever during their acute illness (this group most often is comprised of health care workers or persons caring for family members at home), (iii) worked in a laboratory or animal facility where arenaviruses are handled, or (iv) had sex with someone recovering from arenaviral hemorrhagic fever in the last 3 months. Even persons who meet the above criteria most commonly have a disease other than arenaviral hemorrhagic fever, so alternative diagnoses should always be aggressively sought, especially malaria. Recognized direct contact with rodents in endemic areas, including laboratory mice and pet hamsters and guinea pigs if LCMV infection is suspected (83), should heighten suspicion but is rarely noted even among confirmed cases.

LABORATORY DIAGNOSIS

The difficulty in clinical diagnosis makes prompt laboratory testing imperative, especially when arenaviral hemorrhagic fever is suspected. Unfortunately, no commercial assays are available, a situation further complicated by the BSL-4 designation and Select Agent status of many arenaviruses that limit access and research potential even to many legitimate scientists. Various "in-house" assays are performed in a few specialized laboratories. Common diagnostic modalities include cell culture (restricted to BSL-4 laboratories for most pathogenic arenaviruses), the reverse transcriptase polymerase chain reaction, serologic testing by enzyme-linked immunosorbent assay or immunofluorescent antibody assay, and postmortem immunohistochemistry, each with its unique advantages and disadvantages (134). Although extensive standardization and validation of these assays have not been conducted, they generally appear to have high sensitivities and specificities when performed in experienced laboratories (63, 135).

Virus Isolation

The most definitive and perhaps sensitive diagnostic technique is cell culture. Serum is the most reliable sample to test, but virus can be variably isolated from throat washings, urine, CSF, breast milk, and various other tissues (103, 136–140). A particular advantage of cell culture is that, with appropriate antiserum, it enables detection of virtually any strain of virus. However, the time required for virus propagation and the need for a BSL-4 laboratory render cell culture more of a confirmatory test and research tool. Cell culture at higher dilutions may yield a positive result from a specimen found negative at lower dilutions.

Nucleic Acid Detection

RT-PCR has become an increasingly valuable tool for the VHFs, capable of detecting Lassa virus in over 80% of cases in the first 10 days of illness (18, 141, 142). The technique can be performed in hours, appears to be at least as sensitive as cell culture, and has the significant advantage of not requiring any reagent that must be produced in a BSL-4 laboratory. Since high viremia (or viral RNA copy number or ELISA antigen as surrogates) correlate with death, quantitative RT-PCR also provides prognostic value (56, 63). Sequence heterogeneity of Lassa viruses across West Africa has traditionally posed a challenge to PCR-based diagnostics due to primer-target mismatch but recent development of assays targeting conserved portions of the Lassa virus GPC or arenavirus L genes may have resolved this problem (142, 143). Real time and multiplex PCR assays have been developed that may vastly improve the rapidity and ease of diagnosis of Lassa fever and the many diseases in its differential diagnosis (144–147).

Due to the extreme sensitivity of RT-PCR, contamination and false-positive results are a real concern, especially when the assay is performed in more rudimentary facilities in resource-poor areas where separate spaces for pre- and post-PCR procedures and the routine use of positive and negative controls are not always possible. In the worst case, outbreaks or even bioterrorism could be falsely suspected. The use of one-step assays, sequencing of PCR products to distinguish

them from reference strains, targeting different portions of the genome, and use of multiple supporting diagnostic methods can minimize the risk of false positives (146). False-negative RT-PCR results in VHF are also a concern, which may be due to inhibition by substances circulating in blood, perhaps released by tissue damage or during the RNA extraction process (143, 148). Appropriate inhibition controls must be included in all assays. False-negative results may also occur when patients are tested very early in the course of disease before viremia reaches the threshold of detection for the assay. In this case, if arenavirus infection is still suspected on clinical grounds, the patient should be managed empirically as for VHF and retested in 48 hours when viremia should logically have risen above the detection threshold. Serologic detection of IgM antibody (see below) is also of use in these cases.

Serologic Assays

In-house ELISA and IFA assays for arenaviral antigen and IgM and IgG antibody have been developed over the years and have been a mainstay of diagnosis, although now the antigen assay is increasingly replaced by RT-PCR (63). Since antigen and IgM antibody detected by ELISA are not typically seen at the same time in the course of the disease, both components are necessary. ELISA assays are high throughput and can be performed with inactivated specimens using standard equipment present in many diagnostic laboratories. ELISA testing for Lassa fever appears to have a sensitivity and specificity over 90% when antigen and IgM antibody assays are conducted in tandem (63, 135). Detection of antibody through IFA can also be a valuable tool, but it is not as routinely sensitive or specific and is more subjective in its interpretation, varying with the experience of the technician (63, 64).

In survivors, IgM antibodies begin to appear about a week after disease onset and progressively increase as virus clears, lasting at least some months (63). There are conflicting reports regarding the timing of IgM antibody appearance; this discrepancy may reflect differences in the production of reagents and their target epitopes, variations in assay sensitivity and specificity, and experience of the persons conducting the assay (63, 103, 149). IgG antibody begins to appear 2 to 3 weeks after onset and recovery and lasts for years. Antibody has been found in persons who left the Lassa fever endemic area over 40 years earlier and had no opportunity for reexposure (150).

Although ELISA assays have the advantage of being high throughput and less prone to false positives due to contamination than RT-PCR, the lack of commercially available reagents is a major drawback to their development; both ELISA and IFA traditionally rely on antigen produced through virus propagation in cell culture in BSL-4 laboratories, which poses a major barrier to production given the scarcity of such laboratories. Cross reactions between antigenically similar arenaviruses may also pose a problem with the serologic assays, although sometimes can be resolved by neutralization testing (74). Various recombinant-protein and virus-like particle-based assays are being developed that may eventually relieve the diagnostic bottleneck, as well as improve sensitivity and specificity, although interpretation and validation of these assays is proving challenging (151, 152).

Immunohistochemistry

Postmortem diagnosis of some VHFs may be established by pathology examination with immunohistochemical staining of formalin-fixed tissue, but the assay does not appear to be as reliable for arenavirus infection as for some of the other VHFs (153).

PREVENTION

Patient Isolation and Infection Prevention and Control

All patients with a clinically compatible syndrome should be presumed infectious and kept under "VHF isolation precautions" (including use of mask, double gloves, gown, protective apron, face shield or goggles, and boots or shoe covers) until a specific diagnosis is made (154, 155). Powered air-purifying respirators and other small particle aerosol precautions should be used when performing procedures that may generate aerosols, such as endotracheal intubation and sample centrifugation. If available, placement in a negative airflow room is prudent, but hermetically sealed isolation chambers are not required and may have severe adverse psychological effects on both patient and staff. Access to the patient should be limited to a small number of designated staff and family members with specific instructions and training on the implementation of VHF isolation precautions. Protective measures for agricultural workers, field biologists, pest control and pet store workers, and pet owners who may face occupational exposure to rodents and their excreta are reviewed elsewhere (1, 33, 156).

Disinfection

When contamination is suspected, such as in homes or hospitals treating persons with VHF, disinfection is effective. Sodium hypochlorite (i.e., household bleach) is the most readily available effective inactivation method (154). Bleach solutions should be prepared daily; starting with the usual 5% chlorine concentration, a 1:100 (1%) solution should be used for reusable items, such as medical equipment, patient bedding, and reusable protective clothing before laundering. A 1:10 (10%) bleach solution should be used to disinfect excreta, corpses, and items to be discarded. Workers cleaning areas potentially contaminated by the excreta of small mammals should let the area aerate before entering, then spray the area with the 10% bleach solution and let it sit on the surface for at least 15 minutes before mopping or wet sweeping (157). A site with appropriate security should be dedicated for waste disposal if routine autoclaving is not available.

Specific guidelines exist regarding handling and burial of corpses of victims of VHF (154). The question of the safety of exhuming remains of persons who died of arenaviral hemorrhagic fever overseas for transport to their native home occasionally arises. No data regarding the viability of arenaviruses under these circumstances are available, but it is unlikely that the viruses would survive long under the harsh pH conditions of a decomposing corpse unless the ambient temperature is below freezing, which is unlikely in the endemic areas of most arenaviral diseases. If corpses are to be exhumed, the same protective measures used for burial of victims of VHF, including placing the cadaver in a sealed body bag, should be used.

Vaccines

In Argentina, use of a live attenuated vaccine called "Candid #1" began in 1991, decreasing the incidence of Argentine hemorrhagic fever from 837 cases that year to an average of 122 cases yearly from 1992 to 2009 (158). The vaccine may also protect against Bolivian hemorrhagic fever,

although it does not appear to cross-protect against other arenaviruses (159). However, "Candid #1" is generally not available or approved outside of Argentina, and even within Argentina supplies are insufficient to cover the population at risk. Furthermore, despite the efficacy and generally excellent safety profile of "Candid #1" for Argentine hemorrhagic fever, fear of reversion to virulence with live-virus vaccines is a major disincentive to exploring its application to other arenavirus infections.

Other arenavirus vaccines are in developmental stages. A number of vaccine platforms have been explored in animal models of arenaviral hemorrhagic fever, primarily aimed at preventing Lassa fever. Approaches include inactivated or attenuated viruses (160, 161), recombinant vaccinia viruses (117), RNA replicon vectors derived from an attenuated strain of Venezuelan equine encephalitis virus (162), recombinant salmonella typhimurium (163), arenavirus protein subunits (164), naked DNA (165), chimeric viruses using the yellow fever 17D vaccine strain (166, 167), reassortant Lassa/Mopeia viruses (168–170), virus-like particles (171), and recombinant vesicular stomatitis virus (VSV) (172, 173). The arenavirus GP1 appears to be the most important immunogenic protein.

The recombinant VSV platform is perhaps the most promising candidate, providing 100% protection after a single dose in a monkey model of Lassa fever (174). This vaccine may be effective when given by the nasal or oral route, potentially facilitating its use in epidemics or as postexposure prophylaxis (175). A vaccine for Ebola virus using this same platform has recently been shown to be safe and effective (176). However, the social, economic, and political barriers to development of arenavirus vaccines are perhaps at least as formidable as the scientific ones, considering that most arenavirus diseases exist in geographically restricted areas with resource-poor populations.

Postexposure prophylaxis

Although data from clinical trials are sparse, most arenaviruses show *in vitro* and *in vivo* sensitivity to the nucleoside analogue antiviral drug ribavirin. However, given the low secondary attack rate of arenaviruses, postexposure prophylaxis is rarely indicated (177–179). Furthermore, there are no data on the efficacy, dose, or duration of administration of ribavirin for this purpose. Postexposure prophylaxis with oral ribavirin should be considered only in cases of direct unprotected contact with blood or bodily fluids from a person with confirmed or highly suspected arenaviral hemorrhagic fever (Table 3). Persons who develop manifestations of VHF should be immediately converted to the IV form. Prophylaxis should not be given if the only exposure was during the incubation period. Specific guidelines have been published (180).

Outbreak Management

Control of most VHFs relies on classic public health principles of identification and isolation of cases and monitoring of their contacts. However, the early nonspecific presentation of arenavirus infection poses a serious challenge to case identification. Fortunately, the low secondary attack rate affords a measure of reassurance even when cases go unrecognized as long as proper barrier nursing is maintained. Furthermore, since mild cases are not very infectious, missed or delayed diagnosis of these patients is unlikely to pose a problem from an infection control standpoint.

Persons with unprotected direct contact with a patient during the symptomatic phase of arenaviral hemorrhagic fever should be monitored daily for evidence of disease for three weeks (the longest known incubation period) after their last contact. Contacts should check their temperature daily and record the results in a log. Despite the lack of evidence for transmission during the incubation period, exposed persons should avoid close contact or activities with household members that might result in exposure to bodily fluids, such as sexual contact, kissing, and sharing of utensils. Hospitalization or other confinement of asymptomatic persons is not warranted, but persons who develop fever or other signs and symptoms consistent with VHF should immediately be isolated until the diagnosis can be excluded.

Rodent Control

In the absence of effective vaccines for most arenavirus infections, effective and sustainable control relies on avoidance of known reservoir host habitats or, when this is not feasible, implementation of measures to prevent direct contact with rodents and their excreta. Most successful control programs entail principles of integrated pest management, combining biological and chemical control measures with education or regulation to change human behaviors that put them at risk (181, 182). Structural improvements to homes may be important for arenaviruses whose rodent hosts invade houses (183).

For LCMV, Lassa, and Machupo viruses, whose hosts often colonize human dwellings, measures to improve "village hygiene" are advocated to discourage rodent invasion, including assuring proper waste disposal, eliminating unprotected storage of garbage and foodstuffs, reducing clutter and vegetation around houses that give rodents shelter, and plugging holes that allow entry into homes (184). These measures may not always be possible with the rudimentary construction of houses in some regions, especially with regard to Lassa fever in areas of West Africa that have been ravaged by war or civil unrest.

Although complete elimination of rodents from the environment through trapping or poisoning is not feasible or

TABLE 3 Ribavirin therapy for Lassa fever

Indication	Route	Dose	Interval
Treatment	IV*	30 mg/Kg (maximum 2 g)**	Loading dose, followed by:
	IV*	15 mg/Kg (maximum 1 g)**	Every 6 hrs for 4 days, followed by:
	IV*	7.5 mg/Kg (maximum 500 mg)**	Every 8 hrs for 6 days
Prophylaxis	PO	35 mg/Kg (maximum 2.5 g)**	Loading dose, followed by:
	PO	15 mg/Kg (maximum 1 g)**	Every 8 hrs for 10 days

IV, intravenous; PO, oral administration.
* The drug should be diluted in 150 ml of 0.9% saline and infused slowly.
** Reduce the dose in persons known to have significant renal insufficiency (creatinine clearance of less than 50 ml/min).

desirable, considering their importance to overall healthy ecosystems, short-term intensive rodent elimination in defined areas may occasionally be useful to control outbreaks. One outbreak of Bolivian hemorrhagic fever in 1963 to 1964 in the village of San Joaquin ended abruptly after 2 weeks of continuous trapping in homes during which 3,000 big laucha were captured (185, 186). However, a single trapping session in houses in Sierra Leone did not diminish the incidence of human Lassa virus infection (184). A sustained elimination program would be necessary for effective long-term control because rodents from surrounding fields and forests may quickly recolonize villages and homes. Rodent elimination programs are unlikely to control transmission of Junín and Guanarito viruses to humans because their reservoir hosts are widespread in agricultural fields, although targeting the linear habitats preferred by drylands vesper mice might be plausible (1). Crop replacement or periodic burning of tall grassy areas that are in close proximity to agricultural fields and human habitation have been recommended to control these viruses, although the measures are untested (187).

TREATMENT

General supportive measures

When possible, patients with VHF should generally be treated in intensive care units since severe microvascular instability, often complicated by vomiting, diarrhea, and decreased fluid intake require continuous hemodynamic and electrolyte monitoring and aggressive fluid replacement (188). Overaggressive and unmonitored rehydration may lead to significant third-spacing and pulmonary edema. Fluid and blood pressure management guidelines for septic shock are recommended for VHF, although there are no efficacy data on their use for this condition (127). IV fluids, blood products, and vasopressors may be indicated. Although disseminated intravascular coagulopathy is not frequently noted in arenavirus infection, the possibility merits monitoring the relevant laboratory parameters if bleeding and thrombocytopenia persist, requiring transfusion of platelets or fresh frozen plasma. Vitamin K (10 mg on 2 consecutive days) may be given, especially if underlying malnutrition or liver disease is suspected. Until the diagnosis of arenavirus infection is confirmed, patients should be immediately treated with appropriate broad spectrum antibacterial or antiparasitic therapy, with specific consideration of antimalarial agents for malaria and doxycycline for tick-borne rickettsial diseases. Secondary bacterial infection should be suspected if patients have persistent or new fever after about two weeks of illness.

Acetaminophen (500 to 1,000 mg q4 to 6 hr), tramadol (50 to 100 mg q4 to 6 hr), opiates, or other analgesics should be used for pain control, but salicylates and nonsteroidal anti-inflammatory drugs should be avoided due to the risk of bleeding. Prophylactic therapy for stress ulcers with H_2 receptor antagonists (e.g., ranitidine 50 mg IV every 8 hr) is appropriate. Seizures can usually be managed with benzodiazepines or phenytoin, with careful attention to possible respiratory depression. The use of sedatives and neuromuscular blocking agents should be minimized, but haloperidol (0.5 to 5 mg two or three times daily) or a benzodiazepine (e.g., lorazepam 1 to 10 mg orally daily in 2 to 3 divided doses) may be used. Impaired gas exchange is not typically a prominent feature of arenavirus infection, especially in the absence of iatrogenic pulmonary edema. Intubation and mechanical ventilation should be avoided if possible because of the risk of barotrauma and pleural and pulmonary hemorrhage. Uterine evacuation appears to lower maternal mortality and should be considered in pregnant patients, although performed with extreme caution as this can be considered a high-risk procedure with regard to potential nosocomial transmission (130).

Antiviral therapy

Ribavirin

Ribavirin should be considered in all cases of arenaviral hemorrhagic fever. In Lassa fever, IV ribavirin has been shown to decrease mortality of severe disease from 55% to 5% when begun within the first 6 days of illness (Table 3) (178). The mechanism of action is unknown, although lethal mutagenesis has been proposed (180). Although few data are available, oral ribavirin may also be effective in some cases, but less so than the IV form, most likely because the serum concentration achieved through oral administration is on the borderline of the mean inhibitory concentration of ribavirin for most arenaviruses (178, 179, 189). Absorption of oral ribavirin from the gut may also pose a barrier given the vomiting and diarrhea often present in VHF. Until more data are available on the efficacy of oral ribavirin, the entire treatment course of ribavirin should be administered IV (Table 3).

Major adverse effects due to short-term ribavirin are rare. The primary adverse effect is a dose-dependent, mild-to-moderate hemolytic anemia that infrequently necessitates transfusion and disappears with cessation of treatment (95, 178, 190, 191). Rigors may occur when ribavirin is infused too rapidly. Relative contraindications include severe anemia or hemoglobinopathy, coronary artery disease, renal insufficiency, decompensated liver disease, breast feeding, and known hypersensitivity. Although findings of teratogenicity and fetal loss in laboratory animals have rendered ribavirin technically contraindicated in pregnancy (pregnancy category X), its use must still be considered as a life-saving measure given the extremely high maternal and fetal mortality associated with arenaviral hemorrhagic fever in pregnancy.

Hemoglobin, hematocrit, and bilirubin levels should be checked at initiation of ribavirin therapy and then every few days, with consideration of transfusion of packed red blood cells if significant anemia develops. Because of the long terminal half-life ($\sim$24 hours) and large volume of distribution, ribavirin may still have effect for a time even after cessation, particularly in red blood cells where it accumulates.

Convalescent Plasma

Convalescent plasma is efficacious in Argentine hemorrhagic fever and may be indicated, when available, in other New World arenavirus infections. However, in Argentine hemorrhagic fever it has been associated with a convalescent-phase neurologic syndrome characterized by fever, cerebellar signs, and cranial nerve palsies in 10% of treated patients. The complication has not been seen in other arenavirus infections. Limited observations in humans and animal studies suggest that convalescent plasma is also efficacious for Lassa fever, but only if the plasma contains a high titer of neutralizing antibody, which is not always the case even in survivors, and there is a close antigenic match between the infecting viruses of the donor and recipient. The late neurologic syndrome has not been reported in patients with Lassa fever, although opportunities for observation and possible detection of this syndrome have been limited. Given the logistical

challenges inherent in the use of immune plasma, including risk of concomitant transmission of other blood-borne pathogens and lack of an existing bank of immune plasma for this purpose, this therapy should be reserved for severe and refractory cases unresponsive to ribavirin or when ribavirin is not available.

Experimental Therapies

Antivirals

A number of experimental antiviral therapies for arenavirus have shown activity *in vitro* and *in vivo*, including small molecules (192–194), nucleoside analogs (195, 196), inhibitors of S-adenosyl-l-homocysteine hydrolase, and tyrosine kinase inhibitors, but are not yet ready for clinical application (197). Transcriptome profiles from monkey models of arenavirus infection may aid in identifying key genes, some of which are the targets of drugs already in clinical use, and thus could potentially be used "off-label" for arenavirus infection, perhaps, depending on the mechanism of action, in combination with ribavirin (198, 199). Ribavirin combined with IFN alfacon-1, a consensus IFN, diminished mortality and disease severity in a hamster arenavirus model (200). Although approved for clinical use in humans, IFN aflacon-1 has not been tested in human arenavirus infection, perhaps in part due to its high cost, systemic toxicity, and need for repeated doses.

Host-Directed Therapies

Interest is increasing in approaches that would mediate the underlying pathogenesis of VHF and thus have efficacy regardless of the specific etiologic virus. These include immune and coagulation modulators. However, although yet to be systematically tested in humans with arenaviral hemorrhagic fever, trials of various immune modulators in septic shock, including ibuprofen, corticosteroids, anti-TNF-α, nitric oxide inhibitors, statins (HMG-CoA reductase inhibitors), and interleukins, have not shown conclusive benefit. Other immunomodulating approaches being explored include enhancing immune recognition of infected cells and dampening immune responses through the blockage of toll-like receptors (197).

An experimental recombinant inhibitor of the tissue factor/factor VIIa coagulation pathway, rNAPc2, decreased mortality in Ebola virus–infected monkeys and has completed a Phase I trial in humans, but has not yet been tried in arenavirus infection. Data on the use of other anticoagulants, such as heparin sulfate, antithrombin III, recombinant factor VIIa, and tissue-factor pathway inhibitor to treat VHF are either nonexistent or inconclusive. Until more efficacy and safety data are available, neither immune nor coagulation modulating drugs can be recommended for arenavirus infection.

Criteria for Hospital Discharge

In the past few years the relative widespread availability and ease of RT-PCR testing has prompted the use of laboratory, as opposed to clinical, discharge criteria for many VHFs (201). Common criteria are to require two RT-PCR negative blood tests at least 72 hours apart. However, the matter remains up for debate; for almost half a century before the availability of RT-PCR testing in West Africa, patients with Lassa fever were routinely discharged when considered to be clinically recovered, with no reports of further virus transmission with the exception of rare cases of presumed male-to-female sexual transmission. This empiric observation is not surprising, considering the well-established direct relationship between level of viremia (and thus infectivity), and the clinical status of the patient. Furthermore, due to logistical and biosafety concerns, most RT-PCR results are usually not confirmed with cell culture, making it difficult to determine whether a positive RT-PCR indicates the presence of infectious virus, especially when cycle thresholds (which are inversely related to level of viremia) are high. Furthermore, swabs of other body fluids and surfaces of VHF survivors may continue to test RT-PCR positive even after the virus has been cleared from the blood. More thought and research is clearly needed to develop evidence-based discharge criteria that incorporate both laboratory results as well as knowledge gained from decades of empiric observation.

Management of Convalescence

Clinical management during convalescence includes the use of warm packs, acetaminophen, nonsteroidal anti-inflammatory drugs, cosmetics, hair-growth stimulants, anxiolytics, antidepressants, nutritional supplements, and nutritional and psychological counseling as indicated.

Because of the potential delayed clearance of virus from the urine and semen, abstinence or condom use is recommended for 3 months after acute illness. Transmission through exposure to urine or feces during convalescence has not been noted, but simple precautions to avoid contact with potential excretions in this setting are prudent, including separate toilet facilities and regular hand washing. Breastfeeding should be avoided during convalescence unless the breast milk can be tested and determined to be negative or there is no other way to support the baby.

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Enteroviruses

JOSÉ R. ROMERO

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VIROLOGY

Taxonomy

The genus *Enterovirus* (EV) was so designated because its members replicate primarily in the human gastrointestinal (GI) tract. The original taxonomic classification of the EVs recognized 64 prototype serotypes within the family *Picornaviridae* ("pico" meaning small, "rna" for ribonucleic acid genome) (Table 1) (1, 2). Additional serotypes continue to be discovered, and the original genus currently has more than 100 confirmed serotypes (3, 4). The "traditional" species designation within the genus includes the polioviruses (PV), coxsackieviruses A and B (CV-A and CV-B, respectively), echoviruses (E), and the "numbered" EVs (Table 1). The original speciation of the EVs was based on the ability of individual serotypes to grow in various cell cultures and produce disease in animal systems (1).

More than a century ago the PVs were the first of the EVs identified as a result of their pathogenicity in humans (5). In animal models they could produce flaccid paralysis in monkeys but failed to induce disease in murine systems. With the advent of tissue culture, they were found to replicate prolifically in cells of human and simian origin. The coxsackieviruses followed as the next species within the genus to be identified. The CV-A serotypes characteristically were capable of replication in suckling mice, resulting in a diffuse myositis and flaccid paralysis (6). They did not readily replicate in cultured simian- or human-derived cells. In contrast, CV-Bs grew readily in tissue cultures of both simian and human origin as well as in suckling mice (7). Their pathology in suckling mice differed from type A serotypes in that the myositis was more focal, and direct infection of brain, myocardium, pancreas, and liver also occurred. Neither the CV-As nor CV-Bs were pathogenic for monkeys. The echoviruses (**e**nteric **c**ytopathic **h**uman **o**rphan viruses), so designated because of their initial apparent lack of association with human disease, were defined by their ability to grow well in simian-derived tissue culture cells but not at all in suckling mice and monkeys (8). The original distinctions based on cell culture and animal systems became significantly blurred as new strains were identified and as use of new cell lines resulted in crossover patterns of EV growth. For that reason, serotypes reported from 1973 to date have been designated as "numbered" EVs (enteroviruses 68, 69, etc.) (9).

Assigning a new serotype within each of the EV species was based on generating antisera against the new serotype and reciprocal cross-neutralization testing using a complete panel of prototype strains and antisera (10). Even using such an approach misclassification occurred, requiring reclassification of several of the original EV serotypes. Coxsackievirus A23 and E-8 were found to be identical to E-9 and E-1, respectively. Echovirus 10 and E-28 have been reclassified as reovirus 1 and human rhinovirus 1A, respectively.

The advent of molecular virology opened the way for further refinement of definition of the genus *Enterovirus*, its species and constituent serotypes (3, 4). Hepatitis A virus (previously EV 72) was moved into a new genus, *Hepatovirus*. Sequence analysis of echoviruses 22 and 23 has demonstrated them to be genotypically distinct from other members of the genus (11) and has led to their reclassification into a genus unto themselves, designated *Parechovirus* (12). Phylogenetic analysis using the complete or partial coding sequence of VP1, the major EV capsid protein, has been found to be extremely useful in defining EV taxonomy at the species and serotype levels (3, 13, 14). Using this approach, it was recognized that several EVs originally accepted as individual serotypes were, in fact, strains of one another (CV- A11 [prototype strain Belgium-1] and CV-A15 [prototype strain G-9], CV-A13 [prototype strain Flores], and CV-A18 [prototype strain G-13]) (13).

Criteria for species demarcation within the genus *Enterovirus*, based on molecular and biologic characteristics, have been issued by the International Committee on Taxonomy of Viruses (3). Criteria currently in use for species demarcation of the EV require that members of an EV species share a amino acid identity of >70% in the polyprotein, 60% in the capsid P1 coding region, and 70% nonstructural proteins 2C plus 3CD; share a limited range of natural hosts and host receptors; possess a genome base composition of guanine and cytosine that varies by no more than 2.5%; and share a significant degree of compatibility in proteolytic processing, replication, encapsidation, and genetic recombination. Based on these criteria, EVs that infect humans are now classified into four species, *Enterovirus* A through D. This has resulted in a redistribution of the original EV into these new groupings (Table 1). Another result of the use of molecular phylogeny for the classification of the EVs has been the reassignment of the rhinoviruses as species within the genus *Enterovirus* (*Rhinovirus* A–D) rather than an independent

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TABLE 1 The Genus Enterovirus- Species Infecting Humans (3,4)

Traditional Classification[a]	Revised Classification[a,b]
• **Poliovirus** (3 serotypes)	• **Enterovirus A** (21 serotypes)
Poliovirus 1–3	Coxsackievirus A2-A8, A10, A12, A14, A16
• **Coxsackievirus Group A** (23 serotypes)	Enterovirus A71, A76, A89-A92, A114, A119, A120, A121
Coxsackievirus A1-A22, A24	• **Enterovirus B** (63 serotypes)
• **Coxsackievirus Group B** (6 serotypes)	Coxsackievirus A9
Coxsackievirus B1-B6	Coxsackievirus B1-B6
• **Echovirus** (28 serotypes)	Echovirus 1–7, 9, 11–21, 24–27, 29–33
Echovirus 1–7, 9, 11–21, 24–27, 29–33	Enterovirus B69, B73-B93, B97, B98, B100, B101, B106, B107, B110-B113
• **Numbered Enteroviruses** (4 serotypes)	• **Enterovirus C** (23 serotypes)
Enterovirus 68–71	Poliovirus 1–3
	Coxsackievirus A1, A11, A13, A17, A19-A22, A24
	Enterovirus C95, C96, C99, C102, C104, C105, C109, C113, C116-C118
	• **Enterovirus D** (5 serotypes)
	Enterovirus D68, D70, D94, D111, D112
	• **Rhinovirus A** (80 serotypes)
	• **Rhinovirus B** (32 serotypes)
	• **Rhinovirus C** (55 serotypes)

[a]Coxsackieviruses A15, A18, A23, echoviruses 8, 10, 22, 23, 28, 34, and enterovirus 72 have been reclassified.
[b]The Enterovirus E-H and J species infect bovine, porcine, ovine, and simian hosts. No Enterovirus I species exists due to concern that "I" could be confused with "1."

genus (3, 4). The genus *Enterovirus* currently is comprised of seven species that infect humans (*Enterovirus* A-D, *Rhinovirus* A-C) and five that infect bovine, porcine, ovine, and simian hosts (*Enterovirus* E-H, J). The *Rhinoviruses* will be discussed in a separate chapter.

The use of oral polio vaccine (OPV) strains of PV (Sabin strains types 1–3) has resulted in the emergence of genetically divergent vaccine-derived PVs (VDPV) (15, 16). OPV types 1 and 3 isolates with >1% divergence or type 2 isolates with >0.6% divergence in the nucleotide sequence of VP1 from their respective parental strains are considered VDPVs. Depending on type, VDPVs may diverge 0.6%–16.7% from their parental Sabin strains in the VP1 coding region. Further characterization of the VDPV is based on epidemiologic or clinical characteristics. If community evidence of person-to-person transmission exists they are designated circulating VDPVs (cVDPVs). The cVDPVs arise from recombination between OPV stains and the nonpolio EVs, generally C-species EVs, in nature. Circulating VDPVs (cVDPVs) emerge in areas with inadequate OPV coverage. Strains isolated from individuals with primary immunodeficiencies as a result of exposure to one of the Sabin strains of PV, usually from vaccination, are designated immunodeficiency associated-VDPVs (iVDPVs). VDPV strains isolated from individuals without an immunodeficiency and without evidence of transmission or isolates from the environment in whom the ultimate source was not identified are designated ambiguous VDPVs (aVDPVs).

Viral Structure

As with other members of the family *Picornaviridae*, the EVs are small (30 nm in diameter), consisting of a nonenveloped protein capsid and a single strand of positive (message) sense RNA (17). The encapsulated RNA of the human EVs constitutes approximately 30% of the virion mass (17). The buoyant density of the EVs in CsCl is 1.34 g/cm^3 (3, 17). Because of their lack of a lipid envelope the EV are insensitive to organic solvents. They are insensitive to nonionic detergents. The EVs are stable at an acid pH of 3.0 or lower, a characteristic that allows them to traverse the stomach and gain access to the intestine where they replicate. The EVs are inactivated by heat (>56°C), UV light, chlorination, and formaldehyde. These physical and chemical characteristics confer environmental stability to the EVs, permitting them to survive for days to weeks in water and sewage.

The capsid is comprised of four proteins, VP1, VP2, VP3, and VP4. The amino acid chains that comprise proteins VP1, VP2, and VP3 arrange themselves to form eight-stranded antiparallel β-sheet structures in what has been playfully designated a "β-barrel jelly-roll" (18) (Fig. 1). The amino acids that connect the β-strands and that make up the N- and C-terminal sequences extending from the β-barrel domain provide each EV serotype with its surface topography and unique antigenicity. The amino acid loops that connect the β-strands and C-terminal sequences determine the EV serotype (2). The four capsid proteins assemble to yield a protomer with proteins VP1, VP2, and VP3 exposed on the surface of the virion and VP4 beneath them lacking a surface exposure (Fig. 1). Five protomeric units assemble to form a pentamer. In turn, 12 pentamers assemble to form the mature virion. Thus, each virion is comprised of 60 repeating protomeric units consisting of equimolar amounts of each of the capsid proteins. For all PV serotypes neutralization sites are most densely clustered on VP1, the largest of the capsid proteins and which has the most surface exposed (19).

The atomic structures of several EVs have been resolved and reveal multiple common structural motifs (18, 20, 21). The apex of each pentameric unit (i.e., five-fold axis of symmetry) forms a "star-shaped" promontory or "plateau" surrounded by a deep cleft or "canyon" (Fig. 1). The canyon serves as the viral receptor-binding site for some but not all EVs. At the floor of the canyon is a hydrophobic "pocket" that tunnels toward the five-fold axis. The pocket is occupied by lipids of cellular origin designated "pocket

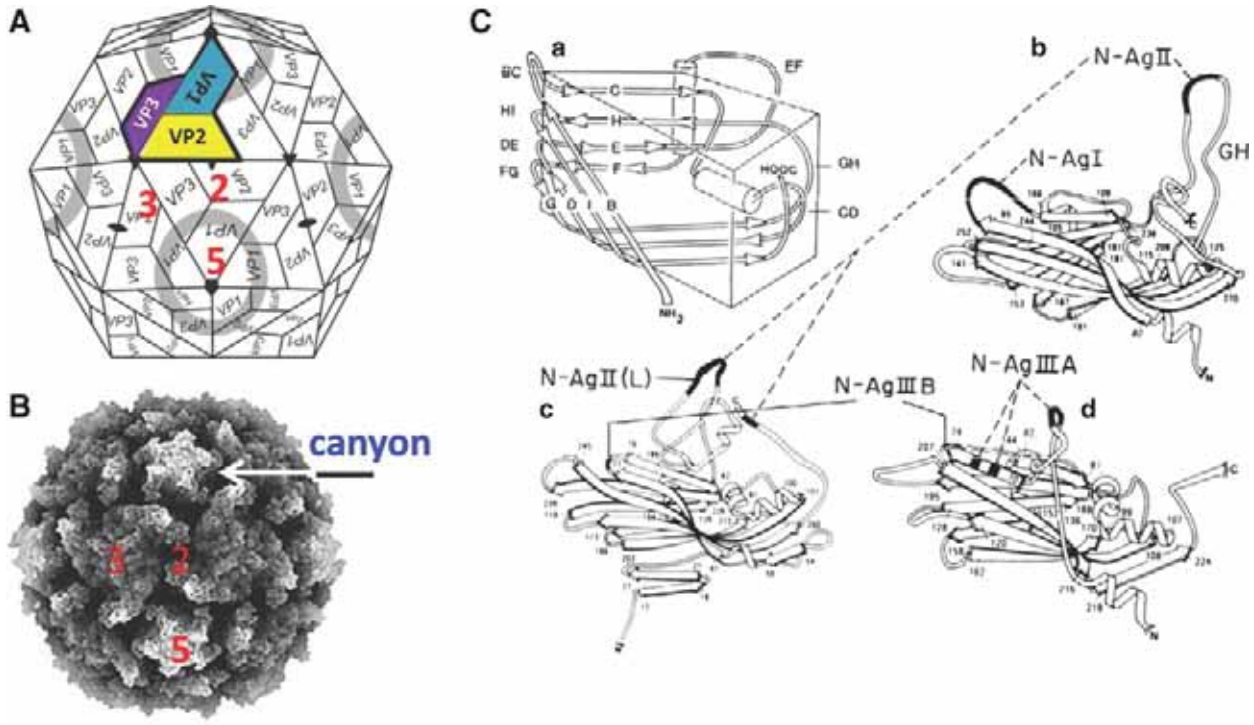

FIGURE 1 **A.** Schematic representation of the icosahedral viral capsid structure of the enteroviruses, as represented by poliovirus. The five-fold (5x), three-fold (3x), and two-fold (2x) axes of symmetry are indicated. The position of one of the 60 repeating protomeric units, each comprised of the surface exposed proteins VP1, VP2, and VP3. **B**. Computer model of poliovirus. The five-fold (5x), three-fold (3x), and two-fold (2x) axes of symmetry are indicated. The canyon is indicated by an arrow. **C.** Schematic representation of the poliovirus proteins. a) Diagram of the structurally conserved core of the 3 major capsid proteins (VP1-3) comprised by an eight-stranded, wedge shaped, antiparallel beta-barrel core. The ribbon diagrams of b) VP1, c) VP2, and d) VP3 are shown with the locations of the major neutralizing antigenic sites (N-Ags) of poliovirus type 1. (Reprinted from Jiang P, Liu Y, Ma HC, Paul AV, Wimmer E. 2014. Picornavirus morphogenesis. *Microbiol Mol Biol Rev* **78**:418–437 with permission.)

factors." At the three-fold axis of symmetry the surface topography of the EV resembles that of a "propeller."

Viral Genome

The RNA genome of the EVs is approximately 7.4 kilobases in length (**Fig. 2**). At the 5′ end of the genome is a virally coded, covalently linked polypeptide, VPg (3B) (2). A long 5′ non-translated region (NTR), approximately one-tenth the length of the total genome, precedes a single open reading frame (ORF) that spans from approximately nucleotides 740 to 7370. The ORF is followed by a short 3′NTR and a terminal polyadenylated (polyA) tail.

The 5′NTR possesses regions of high nucleotide identity in which nucleotide sequences with absolute (or nearly absolute) conservation exist among the EVs (22). These regions have been exploited for the design of primers and probes used for the detection of the EV through nucleic acid amplification (NAA) techniques (i.e., reverse transcription polymerase chain reaction [RT-PCR] and nucleic acid sequence-based amplification [NASBA]) (23). Additionally, internal RNA-RNA interactions within this region result in higher order structures that have essential functions in replication and translation (24–27) of the EV genome. A cloverleaf-like RNA secondary structure formed by the initial 108 nucleotides of the viral genome, designated stem-loop I, is involved in RNA replication (24). A discontinuous region upstream of stem-loop I approximately 450 nucleotides long, designated the internal ribosome entry site (IRES), is essential for translation of the EV RNA genome (25, 26). Lastly, the 5′NTR contains virulence determinants for PV and possibly other EVs (28, 29).

The EV ORF can be subdivided into three regions that code for the viral proteins designated P1-3 (2). The P1 region codes for the four structural proteins (VP1-4) that form the viral capsid. The capsid protein coding sequences are contiguous to one another and are organized 5′ to 3′ as VP4 (1A), VP2 (1B), VP3 (1C), and VP1 (1D) without intervening stop codons (Fig. 2). VP4 and VP2 exist as the precursor VP0 until encapsidation of the viral RNA genome yields the two mature capsid proteins after cleaving of VPO. The largest capsid protein and the one with the most surface exposed is VP1. It contains type-specific epitopes correlating with PV serotypes (3, 13, 30, 31). For all PV serotypes, neutralization sites are most densely clustered on VP1. Additional major neutralizing epitopes have been identified on VP2 and VP3. It is probable that immunodominant epitopes also exist on the VP1 proteins of other EVs. VP1 coding sequences serve as the target for "molecular serotyping" of the EVs (14). Destabilization of VP4, as a result of viral binding to its receptor, results in virus uncoating (32).

The P2 and P3 regions code for seven mature nonstructural proteins (5′- $2A^{pro}$, 2B, 2C, 3A, $3B^{VPg}$, $3C^{pro}$, and 3D -3′) as well as three uncleaved precursor proteins (2BC, 3AB, and $3CD^{pro}$) required for viral protein processing and genome replication (Fig. 2) (2). The proteases $2A^{pro}$, $3C^{pro}$, and $3CD^{pro}$ are required for cleavage of the EV polyprotein.

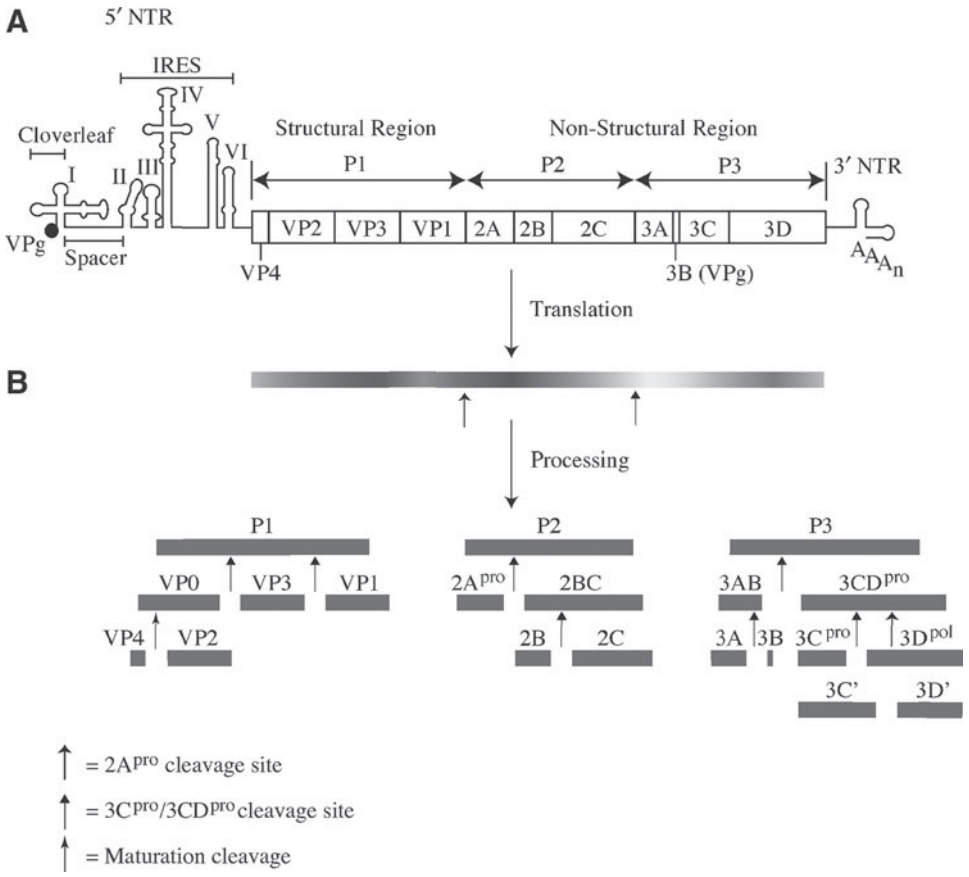

FIGURE 2 Genome organization and translation products of the enteroviruses, as represented by poliovirus **A.** The poliovirus genome. 5′ NTR, 5′ nontranslated region. IRES, internal ribosome entry site. Numerals I–VI represent stem-loop structures within the 5′ NTR. 3′ NTR, 3′ nontranslated region. The poly-A tail at the 3′ end of the RNA is indicated by "AAAn-. **B**. Proteins of poliovirus. Arrows designate site of cleavage and protease involved. (Reprinted from De Jesus NH. 2007. Epidemics to eradication: the modern history of poliovirus. *Virol J* 4:70, Open access article.)

The $2A^{pro}$ also plays a role in RNA replication by stimulating initiation of negative strand synthesis (33). The 2B protein plays a role at an early, as of yet not clearly elucidated, step in viral RNA synthesis. Its synthesis results in inhibition of protein synthesis from the Golgi apparatus and permeabilization of cell membranes. It causes proliferation of membrane vesicles, the site of RNA replication. Protein 2C may have two functions, one as an NTPase and the other directing replication complexes to the cell membranes. It also causes disassembly of the Golgi apparatus as well as the endoplasmic reticulum and formation of vesicles. The 2BC causes membrane permeabilization as well as the formation of vesicles. The protein 3A or VPg is essential for viral replication by acting as the primer for synthesis of the viral RNA genome. Protein 3AB is believed to anchor VPg (3B) to membranes for priming of RNA synthesis. It also interacts with $3D^{pol}$ and $3CD^{pro}$ and may stimulate the polymerase and proteolytic activity of each, respectively. Enteroviruses encode a viral RNA-dependent RNA polymerase designated $3D^{pol}$.

Viral Replication

The viral life cycle begins with attachment of EV to its cellular receptor. Multiple cell-surface molecules have been identified that serve as receptors or coreceptors for the EV (2). For the PV a single protein, CD155, or PVr (PV receptor) is sufficient for cell entry (34). The PV receptor maps to chromosome 19. CD155 is an adhesion molecule and a member of the immunoglobin superfamily that helps to form adherens junctions and is a recognition molecule for natural killer (NK) cells. PVs interact with CD155 via the canyon that surrounds the five-fold axis of symmetry of the virion, where domain 1 of PVr inserts itself.

In the case of other EVs (i.e., CV-B3, CV-A21), interaction with two cell-surface proteins (receptor and coreceptor) may be required for access to the cell cytoplasm. For CV-B3 interaction with CD55, decay-accelerating factor (DAF) (a protein of the complement cascade), is required in order to transport the virus from the apical surface of the cell to the tight junction of the cell (35) where it can interact, via the canyon, with its primary receptor, the coxsackievirus and adenovirus receptor (CAR) (36). Coxsackievirus A21 binds to CD55 but requires ICAM-1 for infection (37). For both CV-B3 and CV-A21, interaction with the primary cellular receptor also occurs at the level of the canyon (38). Coreceptors are believed to be required for several CV-Bs and Es (2).

For CV-A9, interaction with its cellular receptor occurs at a 3-amino-acid stretch (Arg-Gly-Asp) located in a 17-amino-acid extension of the C-terminus of the capsid

protein VP1 (39). However, altering the Arg-Gly-Asp motif does not completely nullify viral infection of cells, indicating that a secondary receptor may be used (40).

Lastly, some EVs may have two or more receptors. EV-A71 has been shown to interact with several cellular proteins: scavenger receptor B2 (SCARB2), P-selectin glycoprotein ligand-1 (PSGL-1), sialylated glycan, heparan sulfate, and annexin II (41, 42). However, binding only to SCARB2 will lead to viral internalization and viral uncoating (42).

The effect of binding of the cellular receptor to the EV canyon has been extensively studied in PV and has led to several models for creation of transmembrane pores that could be used for translocation of the viral RNA across the cell membrane into the cellular cytoplasm (32, 43). Receptor binding to the canyon results in shifts of the capsid proteins VP1, VP2, and VP3 that would allow for extrusion of VP4 and the hydrophobic N-terminus of VP1. Depending on the model, 1) the hydrophobic N-termini of VP1 form a pore traversing the cell membrane, 2) the hydrophobic N-termini of VP1 serve as a membrane anchor while VP4 plays a major role in forming a transmembrane pore, or 3) the hydrophobic N-termini of VP1 act together with VP4 to form a transmembrane pore.

Upon entry into the cell the VPg protein ($3B^{VPg}$) covalently linked to the 5′ end of the viral genome is removed by a cellular protein. The EV 5′- and 3′-NTRs play critical roles in viral regulatory activities such as translation and replication (2). The EVs subvert host cell protein synthesis by inhibiting the formation of the cap ($m^7G(5')ppp(5')N$) binding complex eIF4F (eIF4E + eIF4G + eIF4A) essential for translation of the majority of cellular messenger RNAs (27). The eIF4G is the scaffolding protein with which other proteins or protein complexes (e.g., eIF4A, PAPB, eIF3) interact to form the initiation complex for protein synthesis. The EV protease $2A^{pro}$ cleaves eIF4G so that it cannot interact with the cap-binding protein, eIF4E, and recruit the eIF3-40S ribosomal complex to the mRNA. Within as little as 2 hours after infection nearly all host-cell protein synthesis has been shut down by the EV. In addition to cleavage of eIF4G, $2A^{pro}$ as well $3C^{pro}$ and $3CD^{pro}$ cleave multiple other cellular proteins in order to subvert normal cellular functions for EV translation and replication (44).

Translation of the viral genome is carried out in a noncanonical cap-independent manner and is mediated by the IRES (25, 27). This highly structured region promotes binding of the 40S ribosomal subunit, which then scans to the initiation codon located at approximately nucleotide 740. The model for 40S ribosomal subunit binding to the IRES proposes that EV protease-cleaved eIF4G binds to the IRES. The C-terminal domain of the cleaved eIF4G interacts with the eIF3-40S ribosomal complex, recruiting it to the IRES (27). Additional cellular proteins (La, polypyrimidine tract-binding protein, unr, poly r(C)-binding protein 2 [PCBP2], SRp20) have been shown to be important for IRES function and either bind directly to the IRES or are part of protein complexes bound to the IRES (2, 27, 44).

Translation of the EV genome results in the generation of a single, large polyprotein (Fig. 2) that is immediately posttranslationally processed by the three virally encoded proteases: $2A^{pro}$, $3C^{pro}$, and $3CD^{pro}$. The $2A^{pro}$ and $3C^{pro}$ are active in the polyprotein and release themselves from it autocatalytically. The $3CD^{pro}$ also self-releases from the polyprotein. The primary cleavage between the P1 and P2 regions is carried out by $2A^{pro}$. In addition, it cleaves cellular proteins eIF4GI, eIF4GII, poly A binding protein, dystrophin, and nucleoporins. The $3C^{pro}$ is responsible for the cleavage between 2C and 3A as well as secondary cleavages of the P1 and P2 precursors. The $3CD^{pro}$ is more efficient than $3C^{pro}$ in the cleavage of the capsid proteins contained in the P1 precursor.

Genomic RNA replicates itself tens of thousands of times within each infected cell via the virally encoded RNA-dependent RNA polymerase $3D^{pol}$ (Fig. 2). Synthesis of viral RNA is asymmetric, resulting in a 30- to 70-fold excess of positive sense RNA. Viral proteins $2A^{pro}$, 2BC, 2B, 2C, 3AB, and 3B (VPg) (Fig. 2) are also involved in RNA replication (45). Viral RNA exists in three forms in an EV infection: 1) Single-strand, positive-sense (plus strand) RNA is the most abundant form; 2) Replicative intermediate (RI) RNA consists of full-length plus-strand RNA from which six to eight nascent minus-RNA strands are attached. 3) The replicative form (RF) is double-stranded and composed of one strand each of full-length plus- and negative-RNA strands. EV infection of a host cell results in cytoplasmic accumulation of smooth membrane vesicles as a result of viral proteins, the surfaces of which serve as a platform for viral RNA replication. A ribonucleoprotein complex formed by the binding of PCBP2 and $3CD^{pro}$ to the cloverleaf domain located at the extreme upstream portion of the 5′NTR interacts with polyA binding protein (PABP) bound to the terminal polyA tail of the viral genome (45). This results in circularization of the viral genome template. Binding of $3D^{pol}$, $3C^{pro}$/$3CD^{pro}$, and VPg to a specific viral genomic element (*cis*-binding RNA element or *cre* element) within the 2C protein coding results in uridylylation of VPg (i.e., VPgpUpU). The uridylylated VPg is transferred to the viral genome's polyA tail where it serves as the primer for $3D^{pol}$ to synthesize the minus strand from the plus-strand template. The resultant RNA, known as the RF, is a double-stranded molecule composed of the plus-RNA template and the newly synthesized minus strand. In order to initiate the synthesis of the plus-RNA strand, the RF must be unwound so that the 5′ end of the plus strand can refold into the cloverleaf domain and interact with a protein complex composed of $3CD^{pro}$, PABP, and PCBP2. How this occurs is not yet understood but may involve binding of either $2C/2BC^{ATPase}$, the cellular protein hnRNP C, or 3AB to one or both of the RNA strands of the RI (45). Uridylylated VPg again serves as the primer for $3D^{pol}$ to initiate RNA synthesis of the plus stand of the viral genome. From each minus strand arise several (six to eight) RNA plus strands that create the RI. The nascent plus strands eventually give rise to the full-length plus-strand RNA viral genomes.

Morphogenesis of the virion begins with cleavage of capsid precursor protein P1 from P2 by $2A^{pro}$ (46). The protein $3CD^{pro}$ further cleaves P1 to yield capsid proteins VP0 (the precursor of VP4 and VP2), VP3, and VP1. These proteins self-assemble into a protomer that sediments at 5S in sucrose gradients. Five protomers, in turn, self-assemble to yield a 14S pentamer. Twelve pentamers can assemble to form the 75S empty capsid. One model for encapsidation proposes that positive-sense viral RNA threads itself into the 80S capsid to yield a 150S provirion. In another, more accepted model, the 4S pentamers assemble around the RNA to yield the 150S provirion. In this scenario, the empty 80S capsid may serve as a depot for pentamers. For both models, maturation of the virion occurs with cleavage of VP0 to VP2 and VP4, resulting in an infectious 160S virion, and believed to be mediated by the viral RNA. Cleavage of VP0 stabilizes the virion structure.

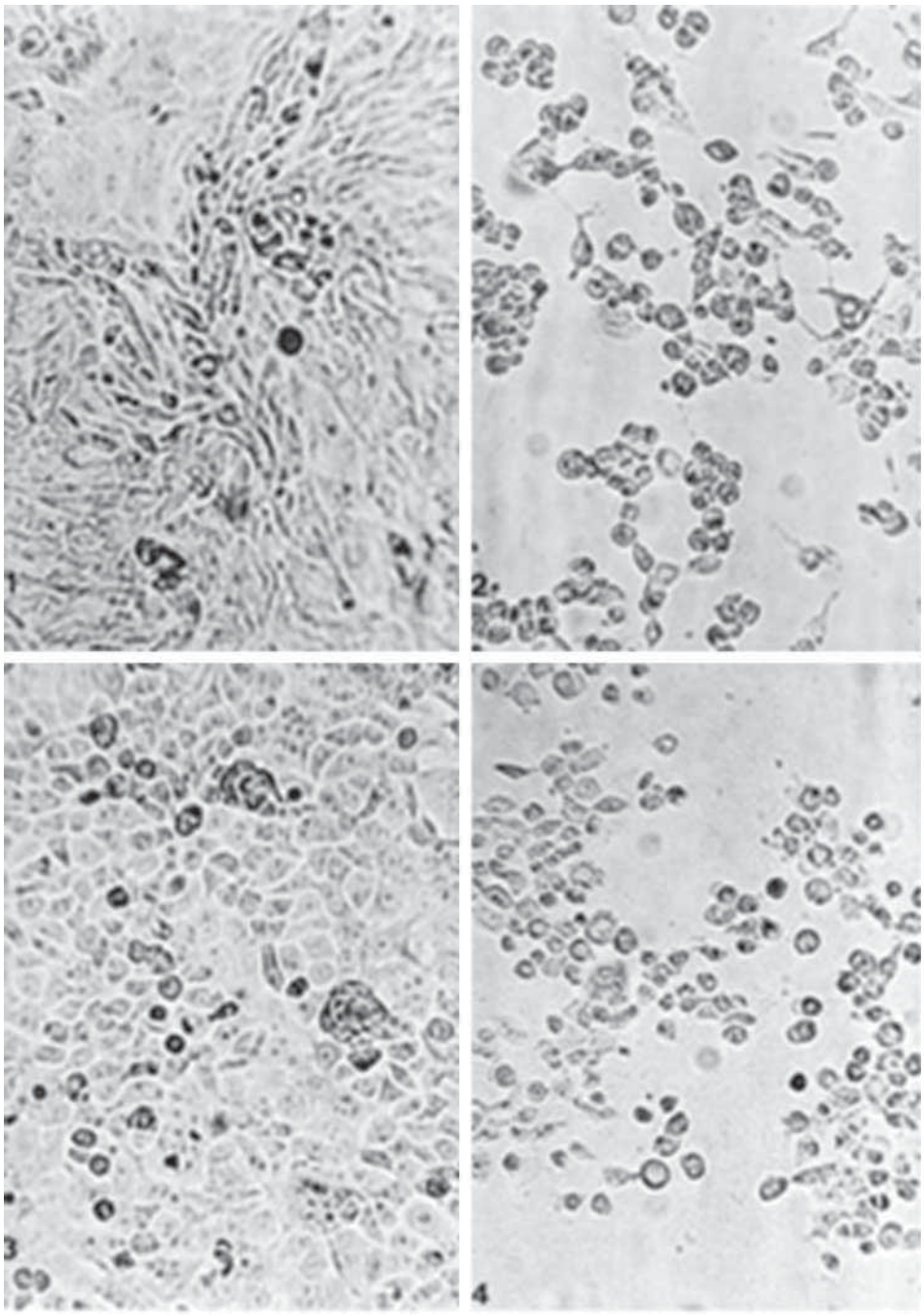

FIGURE 3 Cytopathic effect of poliovirus type 1 infection of tissue culture cells **Panel 1**: Uninfected Rhesus monkey kidney cells. **Panel 2:** Poliovirus-infected Rhesus monkey kidney cells 24 hours after infection. **Panel 3:** Uninfected HEp-2 cells. **Panel 4:** Poliovirus-infected HEp-2 cells 24 hours after infection. (Reprinted from reference 47 with permission.)

Infectious virions are released by cell lysis. The cytopathic effect of the EVs in tissue culture cells has been well described (47) and remains an important diagnostic tool. Light microscopy reveals a characteristic rounding of cells and ultimately detachment from the tissue culture dish (Fig. 3). As seen under electron microscopy, a series of changes occurs, beginning with alteration of nucleus morphology and margination of the chromatin (48). Ribosomes aggregate in the cytoplasm and many clusters of membranous vesicles form; ultimately, the rounded and detached cells lyse.

EPIDEMIOLOGY

Humans are the only natural reservoir for the EVs. The nonpolio EVs are worldwide in their distribution (49–51). With the introduction of polio vaccines, indigenous

transmission of PVs has ceased in all but three countries of the world (Pakistan, Afghanistan, Nigeria) (52, 53). In temperate climates, EV infections appear to have a marked summer/fall seasonality (50). This being said, winter outbreaks of EV disease are well documented (54–56). A high year-round incidence occurs in tropical and subtropical areas with higher incidence during the rainy season (17). The nonpolio EVs are estimated to cause between 10 to 15 million symptomatic infections in the United States each year (57).

The fecal-oral spread of these agents appears to be facilitated, particularly among children, during periods of warm weather. In addition to transmission by direct person-to-person contact, EVs may be recovered from bivalves, houseflies, wastewater, and sewage (58–62). While the role of houseflies in outbreaks has not been established, sewage- and water-associated EV outbreaks have been documented (63–65). The EVs have also been shown to be vertically transmitted from mother to infant congenitally and/or perinatally (2). Rarer routes of transmission include through breast milk and organ transplantation (66–68).

Wild-type PVs, once major contributors to the epidemiologic profile of the EVs in the United States, are no longer causes of acute flaccid paralysis (AFP). However, occasional imported cases of VDPV infection and vaccine-associated paralytic poliomyelitis (VAPP) do occur (69, 70), stressing the need for maintaining immunization against the PVs. Although efforts toward the global eradication of PV infections resulted in the elimination of wild-type infections from the Western Hemisphere in 1991, efforts continue elsewhere in the world (52, 53).

In developing countries the prevalence of paralytic disease remained high (2 to 11 cases/1000 population) throughout the 1970s. In 1988, the World Health Assembly resolved to eradicate poliomyelitis worldwide (52). In 1988, PV was endemic to 125 countries, and in the early 1990s as many as 15,000 cases of paralytic disease were estimated to occur worldwide annually. Despite setbacks (52), continued progress towards total elimination of PV transmission worldwide continues. Transmission of wild-type PV 2 has not been detected anywhere in the world since October 1999 (71). Similarly, there has been an apparent disruption of wild-type PV 3 transmission in Nigeria with no detected cases of AFP since November 2012 (52, 53, 72).

All but two of the six World Health Organization regions (African and Eastern Mediterranean Regions) have been certified polio free. By 2014 indigenous PV transmission had been interrupted in all but three countries, Pakistan, Afghanistan, and Nigeria (52, 53). Encouragingly, no new cases have been identified in Nigeria since July of 2014, suggesting that possible interruption of transmission has occurred (53). The number of cases of AFP due to wild-type PV dropped to 359 worldwide in 2014, with 85% of cases occurring in Pakistan (52). Cases of AFP due to importation of polio to countries previously polio free continue to occur but have decreased as a result of control of disease in endemic countries (53).

An issue encountered as a result of vaccination efforts directed toward global eradication of PV has been the appearance of cVDPVs (15). Factors favoring their development include low OVP coverage and dense populations (73). The public health threat posed by these strains is analogous to that of the wild-type PVs and results in disease indistinguishable from them. The first reported outbreak of poliomyelitis secondary to cVDPV occurred in 2000 on the island of Hispaniola (73). Since then, outbreaks have been reported in 19 countries (15, 16). Approximately 90% of cVDPV outbreaks have been due to strains derived from the type-2 Sabin strain, while type-1 cVDPVs account for approximately 10%. Outbreaks due to type 3-derived cVDPVs are rare (15). In 2014 only four countries reported cVDPV-related AFP cases (74).

Approximately 80 persons with primary immunodeficiencies excreting iVDPVs have been identified worldwide since 1961 when OPV was introduced (15, 16). Type-2 iVDPVs account for 64% of cases, and types 1 and 3 are responsible for the remaining 21% and 15%, respectively. Excretion of iVDPV strains may persist for greater than a decade. From January 2014 through March 2015 aVDPVs were isolated from 16 countries (16).

For the nonpolio EVs, each EV season in each part of the world is dominated by only a few serotypes and, in any given year, multiple EV serotypes may circulate within a community or geographic region (50, 75–81). During the years 1970 to 2008, the predominant nonpolio EV isolates identified in the United States, listed in descending order, were E-9, -11, -30, CV-B5, E-6, CV-B2, CV-A9, E-4, CV-B3, E-7, CV-B4, E-18, CV-B1, E-3, and E-5 (50, 82). These 15 serotypes account for approximately 80% of all EVs reported during that period. The annual determination of predominant serotypes may be influenced by reporting bias, the inability to readily grow certain serotypes in cell culture, particularly the CV-As and the newer EVs, as well as by a skew toward those serotypes causing more serious disease (and hence prompting more laboratory investigation). The predominant serotypes cycle with varying periodicity (75–82), a reflection of the availability of new susceptible host populations, especially children, within a community. Children are the primary victims of EV infections. Data derived from a number of sources spanning the latter half of the 20th and early part of the 21st centuries document that the overall incidence of infections with CVs and Es were highest among children (50, 51, 76, 83).

Because central nervous system (CNS) infections generate the most medical attention among the many manifestations of the EVs, much of the age-related incidence data come from surveys of meningitis and encephalitis. In a Finnish cohort (84), an annual incidence of viral meningitis of 219/100,000 children < 1 year of age was noted, versus 19/100,000 in children between the ages of 1 and 4 years. The vast majority of identified viral pathogens were EVs. In England and Wales the nationwide incidence of EV meningoencephalitis was 313/100,000 in infants < 3 months of age (85). In both reports the incidence dropped with increased age. In Athens, Greece, the annual incidence of EV meningitis was estimated to be 17/100,000 in children < 14 years of age (86). The highest incidence, 26/100,000, was in children 1 to 5 years of age, followed by 24/100,000 in infants < 1 year of age. Past and recent surveys of meningitis and encephalitis have revealed a similar skew toward young infants (51, 87, 88); an incidence peak among young school age children, ages 5 to 10 years of age, has also been reported in several studies (89–91). Occasional outbreaks of EV CNS infections involving predominantly adults occur (54, 92, 93). A surveillance report on causes of viral meningitis among active and reserve U.S. military personnel over a 10-year period documented that the EVs accounted for 86.4% of all confirmed cases. The incidence among active duty members demonstrated lower rates among younger and older personnel (< 20 years of age: 13.1/100,000 person-years; > 40 years of age: 11.7/100,000 person-years) and highest among 25- to 29-year-olds (20.9/100,000 person-years) (94).

A possible explanation for the incidence findings in older children and adults may lie in the recent history of particular serotypes in the geographic area studied. Serotypes with "endemic" patterns, i.e., occurring with significant incidence every year, are most likely to affect only the youngest children (50). They are the most susceptible to these serotypes due to the absence of previous exposure and immunity. Older children and adults are more likely to predominate in an outbreak of a serotype that has not been present in a community for several years, creating a reservoir of susceptible people among children born since the last appearance of that serotype. Although EVs are the most common cause of aseptic meningitis among adults as well, the lower incidence and the greater ease of physical assessment in adults, compared with young infants, reduces the vigor with which a specific viral etiology is sought. When a serotype is introduced into a community that has many susceptible adults, perhaps after a period of absence or because an antigenic variant of a common serotype has emerged, the large number of affected adults will spark renewed interest and increased efforts toward specific diagnosis (51, 92, 93). EV infections other than meningitis and encephalitis are also more commonly identified in children than adults (51, 95, 96), but because of the benign nature of many of these infections, investigations of these other diseases are less commonly performed.

While infection incidence is higher in children, severity of infection as a function of age varies from disease to disease and sometimes from serotype to serotype. Traditional teaching has been that adults suffer the most severe infections with the PVs, manifesting paralytic disease much more commonly than children, in whom asymptomatic infection, aseptic meningitis, or abortive infection are the more common presentations (97, 98). A re-examination of the epidemiology of poliomyelitis, however, finds that the apparent increase in paralytic case:infection ratios with age may actually be the result of increasing immunity within a population following initial introduction of the viruses (99). In PV-naïve populations where poliomyelitis had never appeared, the highest rate of paralysis occurred in the youngest infants (99). Only subsequent to the introduction of PVs, as immunity and hygiene increase in parallel, does the incidence of paralysis appear to increase with increasing age, probably because the actual incidence of susceptible individuals increases with increasing age (99). Similarly, neonates are more likely to suffer severe complications of infections with the Es and CVs than are older children or adults (66, 100, 101). Comparison of case series of adults versus children with aseptic meningitis due the EVs suggests a more severe disease in adults (102, 103); the same is true for pleurodynia (51), an illness usually due to CV-Bs. Myocarditis is most common and most severe in infants < 6 months of age and in young adults; patients from 10 to 19 years of age account for 10% of cases while patients between the ages of 20 to 39 years constitute 52% of cases; the incidence then decreases so that patients over 60 years of age comprise only 5% of all cases of EV myocarditis (104).

Host factors that predispose to or increase the severity of EV infections, other than age and immunodeficiency, have been difficult to identify. Similarly, tonsillectomy and adenoidectomy around the time of PV infection predispose to bulbar poliomyelitis. Perhaps by the same mechanism, physical exercise is an established risk factor for paralytic poliomyelitis (105–107) and a hypothesized one for EV myocarditis (108). A male-to-female incidence ratio for EV infections of 1.4:1 is seen in individuals < 20 years of age (109). The male predominance in cases is not observed in older individuals and may be a reflection of more female caregivers being exposed to the EV. Infection rates with the nonpolio EVs are higher among persons of lower socioeconomic status and in areas of crowding (110). In contrast, poliomyelitis incidence transiently increases with improved societal hygiene as the age of first PV infection is shifted to an older age group. Pregnancy may increase the severity of PV infection and myocarditis (104).

PATHOLOGY

The pathogenesis of EV infections has been studied at molecular, cellular, and organ-system levels (2, 111, 112) and, while much has been learned, much more remains unexplained. The majority of what is known about the pathogenesis of the EV infections in humans has been based on the study of the PVs, the prototypic members of the genus, in experimental infections using chimpanzees more than seven decades ago, and, most recently, from transgenic mice expressing the PV receptor CD155 (112–114). Observations in humans have supplemented these findings.

Pathophysiology

The EVs are transmitted by the fecal-oral route and less commonly by respiratory droplets and transplacentally. The virus may be shed for up to 4 weeks from the nasopharynx and for several weeks to months in the feces. While some replication occurs in the nasopharynx, with spread to upper respiratory tract lymphatics (PV can be isolated from the tonsillar tissue in infected humans), most of the virus inoculum is swallowed (115). The characteristic stability of the EVs at acid pH allows them to traverse the stomach en route to the site of primary infection in the lower GI tract. The site of PV replication in the GI tract is yet to be identified. In human infection, PV has been identified within the ileal wall within Peyer's patches and mesenteric lymph nodes (115). It is believed that the EVs may infect enterocytes of the follicle-associated epithelium (FAE) or M cells within it in the lower GI tract. Supporting this is the finding that CD155 protein has been identified on the surface of FAE, M cells, and in the germinal centers of Peyer's patches (116). Alternatively, M cells have been shown to bind and endocytose PVs, suggesting a similar role in in-vivo infection (117). The M cells could thus transport PV from the gut lumen to cells in Peyer's patch where they then replicate.

A minor (primary) viremia follows replication in the lower GI tract and possibly the nasopharynx, seeding numerous organ systems including the CNS, liver, lungs, and heart. More significant replication at these sites results in a major (secondary) viremia associated with the signs and symptoms of viral infection. If the CNS has not been seeded with the initial viremic episode, spread there may occur with the major viremia. The exact route by which PVs and other EVs gain entry to the CNS is still unclear, but two routes have been proposed and supported by observational and experimental data.

Viremia has been shown to be essential for development of paralytic disease in chimpanzees and supports this route as a mode of entry to the CNS (113). Further support for this mode of access to the CNS comes from studies comparing PV accumulation in the CNS of CD155 transgenic mice and nontransgenic mice (118). Pharmacokinetic analysis of PV injected into these two mice strains indicates that PV is delivered to the brain in significantly greater amounts than would be expected from the vascular concentration. The

mechanism by which the virus gains entrance to the CNS is not known. The mechanism by which EVs leave the blood and enter the CNS is also unknown.

Evidence for access to the CNS via a neural route is available from simian, murine, and human studies. Sciatic nerve inoculation of PV in monkeys results in spread of virus along the nerve and the spinal cord (119). In monkeys and CD155 transgenic mice the initial limb to develop paralysis following inoculation of PV is the one injected (120, 121). Freezing or transsection of the sciatic nerve prevents the development of paralysis of the limb of monkeys or CD155 transgenic mice inoculated intramuscularly with PV (119, 121). Historically, a significant number of children inadvertently inoculated with an incompletely inactivated polio vaccine developed initial paralysis in the limb receiving the vaccine (122). Trauma to a limb is associated with paralysis of that limb following PV infection. This so-called "provocation poliomyelitis" is well described following intramuscular injections into the leg or arm of a patient incubating wild-type PV or those that receive live attenuated PV vaccines (123, 124). Using the CD155 transgenic mice model, it was discovered that the mechanism of provocation poliomyelitis appears to be the induction of retrograde axonal transport (121, 125, 126). Poliovirus may gain access to neurons at the level of the neuromuscular junction, which had been shown to contain CD155 protein (127). Recent evidence also suggests that a CD155-independent uptake mechanism may exist (126). Experimental evidence indicates that intact poliovirions (160S) are transported in endocytic vesicles along the axon by fast retrograde transport (121). Upon arrival at the neuronal body, viral RNA is released and replication ensues (112).

The ability of the EV to replicate in different tissues is not determined solely by the presence of a viral receptor on the cell surface. In humans and CD155 transgenic mice, CD155 expression has been documented in tissues that are not sites of PV replication (34, 128, 129). Although it was initially suggested that the IRES was a determinant of cell tropism for PVs, subsequent experiments failed to support this hypothesis (112). However, the same may not hold true for all the EVs. A chimera containing the 5′NTR of E-12 (Travis strain) in the background of a full-length infectious clone of CV-B3 failed to replicate in murine cells that normally supported the replication of CV-B3 (130). Because the chimera contained the capsid of CV-B3 and its nonstructural proteins, viral entry was eliminated as a replication-limiting factor. This was supported by the finding that the chimera replicated with equal efficiency to that of CV-B3 in HeLa cells. Using reverse genetics, the block to replication in murine cells was localized to a specific stem-loop structure within the 5′NTR, indicating that for the nonpolio EV the 5′NTR may be a determinant of tissue tropism.

Experimental evidence indicates that for the PVs alpha/beta interferon (INF α/β) response may determine cell tropism (131). PV infection of CD155 transgenic mice lacking the INF α/β receptor resulted in viral replication in the liver, spleen, pancreas, and CNS, all of which expressed CD155. Infection of CD155 transgenic mice expressing the INF α/β receptor yielded viral replication in the brain and spinal cord only. In the latter mice, PV infection of extraneural tissues led to rapid and robust production of INF-stimulated genes that limited viral replication in those tissues. In both groups of mice INF-stimulated gene expression in neural tissue was low in the noninfected state and a robust response after infection was not observed. Thus, INF α/β may function as a determinant of cell tropism in CD155 transgenic mice. Further support for this hypothesis was found by documenting that PV-susceptible monkey and mouse kidney cells in culture, as well as primate cell lines, failed to provide a rapid INF response upon infection (132).

The finding that INF α/β is a determinant of PV tissue tropism may provide a long-sought explanation for the relatively rare occurrence of paralytic disease as a result of PV infection (112). In the majority of individuals infected with PV, the INF α/β response limits the replication of PV in extraneural tissues, thereby preventing extension of infection into the CNS. In the $<1\%$ of individuals infected with PV who develop paralytic disease, the INF α/β response may be defective, allowing for significant replication of PV in extraneural tissues that permits CNS infection.

Molecular determinants of pathogenesis are being investigated to understand the clinical phenotypes of specific EV serotypes and subgroups. While all three serotypes of wild-type PVs are known to be neurotropic and neurovirulent, specific tissue tropisms and virulence patterns vary widely among the nonpolio EVs, with certain serotypes consistently reported as causes of specific organ-system disease and others only rarely so. Worldwide, members of the B species of EV are almost uniformly the principal causes of viral meningitis (50, 86, 133). In the United States the serotypes most frequently encountered are, in descending order of frequency, E-9, E-11, E-30, CV-B5, E-6, CV-B2, CV-A9, E-4, CV-B4, E-7, E-18, and E-5 (50). CV-Bs are the most frequent EVs implicated in heart infection (104). Echovirus 11, followed by several other E and CV-B serotypes, are the most important pathogens of neonatal EV sepsis (66, 109); E-11 is also the most common serotype causing chronic meningoencephalitis in antibody-deficient patients (134). Confounding the analysis of genotype-phenotype correlation is the observation that while certain serotypes are more commonly associated with certain diseases, virtually every EV serotype has been associated with virtually every EV disease manifestation.

The determinants of neurotropism and neurovirulence have been investigated extensively for the PVs. Following vaccination with attenuated vaccine strains, reversion to virulence has been observed in the fecally shed virions recovered from normal children (135). The viral RNA of both wild-type and attenuated vaccine strains has been sequenced, and only a few differences exist between them. Comparison of wild-type, vaccine, and revertant PV strains has been extremely useful in identifying neuroattenuating regions of the PV genomes. For the PVs the 5′NTR has been documented to be a major determinant of virulence phenotype (i.e., the ability to cause paralysis) (28). For all three PV Sabin strains the major neurovirulence attenuating sites are clustered within a 10-nucleotide region of the 5′NTR (nucleotides 472–484 relative to type-3 Sabin strain of PV). Additional determinants of virulence are also found in the amino acids encoded in the P1 and P3 coding regions. Neurovirulence-determining genomic regions have yet to be identified for the nonpolio EV.

The search for determinants of virulence in other EVs has been less conclusive. CV-B4 strains with murine pancreatic tropism and virulence are distinguished from avirulent CV-B4 strains by a single amino acid residue in the VP1 capsid protein. The extension of this finding to humans has yet to be established. Genotypic determinants of myocarditis remain to be conclusively identified. One mechanism of CV-induced cardiomyopathy may involve the ability of that virus 2A protease molecule to cleave dystrophin, a cytoskeletal protein found in the heart (136). Using a series of

intratypic capsid and 5′NTR chimeras derived from laboratory and clinical strains of CV-B3 with cardiovirulent and noncardiovirulent phenotypes in mice, a genomic determinant was mapped to the 5′NTR (137). Subsequent evaluation revealed that a higher order RNA structure (stem loop II) within the 5′ portion of the 5′NTR was a major determinant of cardiovirulence in mice (29).

Immune Responses

As with other viral infections, the host immune response to EV infection has both humoral and cell-mediated components (reviewed in 1); the humoral response appears to be the most important. The importance of antibody formation in the clearance and recovery from EV infections is illustrated by the severity and chronicity of EV infections in agammaglobulinemic individuals (134). Similarly, the severity and timing of neonatal EV infections likely reflects antibody deficiency. Poliovirus vaccination has demonstrated the protective benefits of humoral immunity. Anti-EV IgM antibodies are rapidly produced and persist for up to 6 months; IgA and IgG antibodies to the EVs may be detectable for decades following infection. There are four primary antigenic sites on the PVs to which neutralizing antibodies attach (2). Chimeric viruses derived from PVs and CV-B serotypes have been used to show that the BC loop of VP1 (Fig. 1) is an important neutralizing epitope of PV 1 and CV-B4 (31). Epitopes for the nonpolio EVs remain to be determined.

Evidence continues to mount for the important role of innate immunity in EV infections. As discussed previously, INF α/β plays a role in PV infection of mice (131). Toll-like receptor (TLR) 3, which senses double-stranded RNA (dsRNA), was demonstrated to be nearly essential for murine innate response to CV-B3. TLR3-deficient mice had more severe myocarditis and increased mortality when infected with CV-B3 (138). The TLR-3-mediated response may be important in protection of EV myocarditis in humans. Two TLR-3 sequence variants, shown to have reduced responsiveness to dsRNA, have been found in patients diagnosed with EV myocarditis or dilated cardiomyopathy (39). Evidence suggests that increased expression of TLR-8 may be associated with adverse outcomes (heart failure and death) in patients with dilated cardiomyopathy (140).

The study of cell-mediated immune responses to the EVs has been much more limited and the importance of the cellular response in preventing or clearing infection is unclear. T-cell epitopes have been identified on the capsid, near previously discussed B-cell antigens, as well as on nonstructural proteins of CVs and PVs. These studies have been conducted in murine models, and the relevance of T-cell epitopes and lymphocyte responses to human EV infections is still debated. Similarly, EVs and the T-cell responses to them have been implicated in the actual pathogenesis of chronic diseases. Myocarditis, for example, appears to represent an intricate interplay between virus and host genetics/immunity in which direct viral injury (e.g., by dystrophin injury as noted above) and innocent-bystander damage due to the immune response both probably affect the ultimate outcome of disease (1, 136).

Histopathology

The benign nature of the majority of EV infections has resulted in somewhat sparse human pathological data. In patients dying of acute poliomyelitis, mixed inflammatory infiltrates (neutrophils, microglia, and lymphocytes), initially perivascular in location and later in the gray matter, as well as neuronal necrosis and neuronophagia are found from the spinal cord's anterior horn to as far anterior as the hypothalamus, thalamus, and the motor area of the precentral gyrus (141). Outside of the spinal cord the three areas most often severely affected are the reticular formation, vestibular nuclei, and the roof nuclei of the cerebellum. Small hemorrhages and edema are associated with the inflammation. Involvement of the cerebellum, cerebrum, and midbrain may also be found.

Occasional pathologic descriptions of nonpolio EV meningitis, meningoencephalitis, or encephalitis have been reported (142–147). The pachy- and leptomeninges may be edematous with lymphohistiocytic infiltration. In severe cases, there may be edema of the parenchyma and inflammation of the choroid plexus. Perivascular lymphocytic or neutrophilic infiltrates may be present in the meninges or parenchyma. With encephalitis, especially that due to EV-A71, neuronal necrosis, neuronophagia, and microglial nodules may be present. Microscopic hemorrhage, microabscesses, or tissue softening have been reported. In the case of encephalitis due to EV-A71 inflammation of the entire medulla is present to greater or lesser degree. Inflammation in the nuclei of the brainstem and spinal cord is often more severe than that seen in the cerebral cortex or cerebellum.

Histopathological evaluation of the myocardium in cases of acute myocarditis reveals predominantly lymphocytic interstitial infiltrates; however, polymorphonuclear infiltrates of varying degree may also be present (147, 148). The infiltrates may be focal, patchy, or diffuse. Interstitial edema and varying degrees of cardiomyocyte necrosis are common. With resolution, fibrosis may replace most inflammatory changes.

In addition to the findings listed above, infants with severe neonatal EV disease may have involvement of multiple other organs (147, 149). Hepatocellular necrosis, which may range from multifocal to extensive, and hemorrhage are commonly present. Hemorrhagic necrosis of the adrenal glands, renal medullary hemorrhages, pancreatitis, and hemorrhagic pneumonitis may be seen.

Biopsy of the vesicular lesions seen in hand-foot-and-mouth disease (HFMD) demonstrates loose strands of fibrin, lymphocytes, monocytes, and neutrophils within the vesicular fluid (150). The overlying epidermis has extensive acantholysis with reticular degeneration. Edema, perivascular foci of lymphocytes, monocytes, and neutrophils are seen in the upper dermis.

CLINICAL SYNDROMES

Neurologic Illnesses

Poliomyelitis

A total of 90% to 95% percent of wild-type PV infections are asymptomatic. Only 1% to 2% of PV infections during epidemics and <0.1% of infections under nonepidemic conditions result in paralysis. The remaining 4% to 8% of infections result in a flu-like illness termed "abortive poliomyelitis" or the "minor illness." In these patients, fever, fatigue, headache, anorexia, myalgias, and sore throat may last 2 to 3 days followed by complete recovery. Symptoms suggestive of an upper respiratory tract infection (fever, sore throat) are more commonly reported in children (98). Older adolescents and adults may report a "grippe"-like prodrome characterized by fever and generalized aching (98).

The onset of the major illness (i.e., CNS involvement) may be more abrupt in children than in adults (151) and

may follow rather than accompany the flu-like "minor illness." The illness may take on a biphasic appearance, particularly in young infants and children, with "minor illness" preceding "major illness" (98, 151). A biphasic or "dromedary" pattern of fever may occur and is commonly seen in approximately one-third of children. The onset of the major illness in adults may be more gradual and may occur up to 2 weeks from the onset of nonspecific signs and symptoms. Approximately one-third of patients with CNS involvement develop aseptic meningitis ("non-paralytic poliomyelitis") that is indistinguishable from that due to the nonpolio EVs.

Paralysis is often preceded by severe myalgias, more so in adults (98). The pain is most commonly localized to the lower back and the involved limb(s). Meningismus may be prominent. Hyperesthesias and paresthesias may be observed in the same affected muscle groups. Exercise relieves the muscle aches, resulting in anxious movement by affected patients. Loss of superficial and deep tendon reflexes precedes the development of weakness or paralysis. Paresis or paralysis typically appears within 1 to 2 days of onset of myalgias. The risk of onset of paralysis or of its progression continues until the fever subsides.

The paralytic manifestations of PV infection reflect the regions of the CNS most severely affected (98, 151–153). The distribution of the paralysis is characteristically asymmetric with proximal muscles more affected than distal ones and legs more than arms. Single-limb involvement is most common, but quadriplegia may occur. Paralysis of the muscles of the diaphragm may also occur and result in respiratory failure. Paralysis of the bladder and intestinal atony are common.

Cranial nerve involvement may result in so-called "bulbar paralysis" with resultant difficulties in any or all speech, swallowing, breathing, eye movement, and facial muscle movements. Medullary centers controlling respiration and vasomotor function can become involved, with potentially fatal outcome (152). Similarly, involvement of the cranial nerves IX, X, and XII can result in paralysis of the tongue, pharynx, and larynx leading to airway obstruction from secretions.

The natural history of the illness is highly variable, ranging from transient paresis with complete resolution, to rapid progression with complete and permanent paralysis. The short-term outcome, i.e., resolution or paralysis, is evident within several days. The long-term outcome of paralytic poliomyelitis appears to be determined during the first 6 months after onset; absence of improvement during that time period usually suggests permanent paralysis, ultimately, with concomitant limb atrophy and deformity. If improvement occurs, the greatest strength gains occur during the first 6 months (154) and may continue for up to 9 months. The overall mortality of spinal poliomyelitis is approximately 5%. Bulbar and medullary poliomyelitis had high mortality (50% or greater) during the epidemic years in the United States when modern respiratory support techniques were not available (152).

As many as 25% of individuals who recover from paralytic disease may develop the syndrome of post-poliomyelitis muscular atrophy (155). Characterized by recurrent weakness, pain, and atrophy 25 to 30 years after the initial infection, the clinical course is usually a gradual one that seldom results in total disability of the affected areas but does negatively affect quality of life. While viral persistence or reactivation has been postulated, the predominant theory is that the syndrome is a result of aging and neuronal dropout in already compromised neuromuscular connections (156, 157).

Aseptic Meningitis

The clinical disease observed during EV meningitis varies with the host's age and immune status. Neonates are at risk for severe systemic illness, of which meningitis or meningoencephalitis is commonly a part (100, 101, 158, 203). Group B coxsackieviruses were associated with aseptic meningitis in 62% of infants < 3 months of age in one study (100). Echoviruses identified in infants < 2 weeks of age were associated with meningitis or meningoencephalitis in 27% of cases (101). In a prospective study of neonates (< 2 weeks of age) with proven EV infection, 75% had clinical or laboratory evidence of meningitis (158).

The natural history of typical EV meningitis is shown in Fig. 4 (159). Onset is usually sudden, and fever of 38 to 40° C is the most consistent clinical finding, occurring in 76% to 100% of patients (86, 100, 160–164). The fever pattern may be biphasic (100, 159), appearing first with nonspecific constitutional symptoms, followed by resolution and reappearance with the onset of meningeal signs. Nuchal rigidity is found in more than half of the patients, particularly in children older than 1 to 2 years of age (103, 160, 161, 162), but may be less common in infants (160). Headache is nearly always present in adults and children old enough to report it, and photophobia is also common. Non-specific and constitutional signs and symptoms of viral infection, in decreasing order of occurrence, include vomiting, anorexia, rash, diarrhea, cough and upper respiratory findings (particularly pharyngitis), and myalgias (86, 100, 159, 161, 162, 163). Symptoms other than fever may also be biphasic, a presentation observed more often in adults than children.

Aseptic meningitis with certain EV serotypes is associated with particular clinical stigmata, e.g., HFMD frequently occurs with EV-A71 meningitis (164) and rashes are especially common with E-9 meningitis (165), although both incidental findings can occur with numerous other serotypes as well. A history of concomitant family illness, rashes, upper respiratory or GI symptoms, and, occasionally, meningitis, is often obtained (166).

Neurologic abnormalities are unusual; the literature on EV meningitis rarely includes more than 10% of patients with abnormal neurologic examinations. Febrile seizures are among those neurologic "abnormalities" that may complicate aseptic meningitis without implicating parenchymal brain involvement. The syndrome of inappropriate antidiuretic hormone has been reported (167). Additional complications include seizures, coma, increased intracranial pressure (167, 160).

Cytochemical examination of the cerebrospinal fluid (CSF) of patients with EV meningitis usually reveals a modest monocytic pleocytosis (100–1000 cells/mm^3) with normal or slightly depressed glucose levels and normal to slightly increased protein. However, wide variations in these findings have been reported (168), with cell counts ranging from 0 to several thousand, marked hypoglycorrhachia, and markedly elevated CSF protein concentration. Absence of CSF pleocytosis is common in infants (169, 170). Neutrophils may be the predominant cell type in early EV meningitis with a shift to monocyte predominance within 24 to 48 hours (168). Interestingly, CSF eosinophilia has been reported with meningitis due to CV-B4 (171).

The duration of illness due to EV meningitis is usually about 1 week (172), with many patients feeling better immediately after the lumbar puncture (173), presumably due

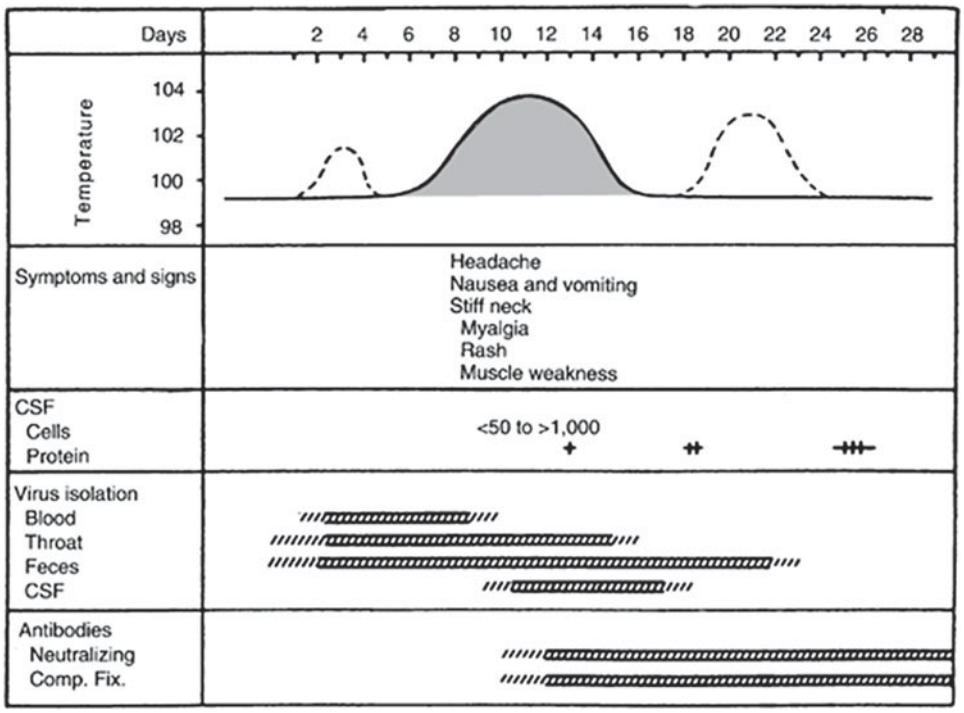

FIGURE 4 The natural history of enteroviral aseptic meningitis. Comp. Fix., complement fixation. (Reprinted from reference 159 with permission.)

to the transient reduction of intracranial pressure with CSF removal. Adult patients often have symptoms that persist for several weeks (162). Comparison of case series of adult EV meningitis (54, 93, 162) with similar series in children (87, 89, 91) suggests that the disease is more severe in adults. Difficulties in eliciting symptoms from young infants may contribute to this impression, in contrast to adults who readily attest to prolonged headaches, dizziness, photophobia, etc.

EV meningitis outside of the immediate (< 2 weeks of age) neonatal period is rarely associated with severe disease or poor outcome. The short-term prognosis of young children with EV meningitis early in life appears to be good; however, there has been some controversy as to possible later sequelae. Uncontrolled studies found numerous subtle long-term behavioral and neurologic abnormalities (174). Neurologic, cognitive, and developmental/language abnormalities have been reported in controlled studies of long-term outcome in children with EV meningitis during infancy (175–180). In the largest and most meticulously controlled study, however, no differences between patients and controls could be demonstrated in any of the neurodevelopmental parameters studied (181). However, the outcomes following EV-A71 CNS infection appear to be different. A study examining the neurodevelopmental outcomes of children with EV-A71 CNS disease found that 5% of children with severe CNS involvement without cardiovascular failure had delayed neurodevelopment (182). In a second study, children with EV-A71 CNS disease exhibited an elevated attention-deficit/hyperactivity disorder-related symptoms, 20% vs. 3% for matched controls (183). Less well studied are the ultimate outcomes of aseptic meningitis in older children and adolescents; preliminary data suggest possible school and learning difficulties, but control patients were not studied (184).

Encephalitis

Encephalitis due to the EVs is well known (185). An 8-year study of 1571 cases of encephalitis identified EVs as the confirmed or possible cause in 4.6% of all cases. EVs comprised 36.5% of viral causes and 29.4% of all etiologies identified (186). Eighty-five percent of isolates serotyped belonged to the Enterovirus B species, 10% to A species, and 5% to C species. In a Spanish study, the EV accounted for nearly 10% of all identified viral causes of encephalitis (133). Children and adolescents accounted for 73%, with a median age of 12 years among confirmed cases. However, in other studies adults accounted for approximately 40% (186).

In contrast to the typical focal disease seen with herpes simplex virus (HSV) encephalitis, EVs have been more commonly associated with global encephalitis and generalized neurologic depression (89, 162, 187, 188). The illness usually begins like aseptic meningitis with a prodrome of fever, myalgias, and upper respiratory symptoms. Onset of CNS signs and symptoms is often abrupt, with confusion, weakness, lethargy, drowsiness, or irritability. Progression to coma or generalized seizures may occur. When meningeal signs and CSF pleocytosis accompany these findings, *meningoencephalitis* is the appropriate term. Unusual but occasional findings include blurred optic discs and other signs of increased intracranial pressure, multifocal encephalomyelopathy, apnea, truncal ataxia, abnormalities of cranial nerves, and paralysis; the latter sign is usually a manifestation of spinal cord involvement and, when accompanying central signs and symptoms, is appropriately termed *encephalomyelitis*.

Focal EV encephalitis is less commonly reported than global disease but may be underappreciated. EVs are demonstrable by brain biopsy in 13% of patients suspected of having HSV encephalitis (189, 190). In a case series and literature review of focal EV encephalitis (191), a variety of focal neurologic findings were seen as well as abnormalities

by imaging studies. Only 28.5% of patients grew EV from cultures of the CSF, reinforcing the insensitivity of cell culture for detection of the EV.

Enterovirus A71-associated rhombencephalitis merits special mention (192) due to its unique epidemiology, clinical presentation, and sequelae. The prevalence of EV-A71 is particularly high in countries of the Asia-Pacific rim where large epidemics, involving hundreds of thousands to millions of individuals, have been reported (165, 193). The syndrome affects principally infants and toddlers. The use of glucocorticoids and/or pyrazolones may be factors for the development of rhombencephalitis (194).

The onset of the rhombencephalitis may follow a biphasic course, during which the onset of the principal neurologic manifestation, myoclonus, is preceded by either hand-foot-and-mouth disease (HFMD) or herpangina (192). Myoclonus associated with tremor and/or ataxia (Grade I rhombencephalitis) comprises the majority of cases. Grade II rhombencephalitis, myoclonus plus cranial nerve involvement, which may include ocular disturbances, or rapidly progressive cardiopulmonary failure following a brief period of myoclonus (Grade III rhombencephalitis) may occur in the remainder of cases. In addition to EV-A71, rhombencephalitis has also been associated with infections due to E-7, CV-A16, CV-B1, and EV-D68 (146, 194a, 195, 196).

Encephalitis due to the EV, and in particular EV-A71, may have more profound acute disease and long-term sequelae. With EV-A71 infections, CNS involvement and brainstem involvement are associated with neurologic sequelae, delayed neurodevelopment, and reduced cognitive functioning (182, 192, 197). However, those with CNS involvement without cardiopulmonary failure did well on neurodevelopment tests.

Individuals with a congenital or acquired absence or deficiency of humoral immunity are at risk for development of chronic CNS or severe disease if infected by EV. Unlike other viruses that are largely controlled by cellular immune mechanisms, the EVs are cleared from the host by antibody-mediated mechanisms. Agammaglobulinemic individuals infected with the EVs may develop chronic meningitis or meningoencephalitis lasting many years, often with fatal outcome (134). This syndrome can also occur in patients with mixed humoral and cellular immune deficiencies such as common variable immunodeficiency and hyperIgM syndrome (198, 199). Although CSF culture-negative periods occur, more consistent evidence of persistent virus has been obtained using PCR (185). Approximately 50% of these infected patients also develop a rheumatologic syndrome, most often dermatomyositis, which is also felt to be a direct result of EV infection of the affected tissues (134). Treatment with antibody preparations intravenously and intrathecally or intraventricularly has resulted in stabilization of some of these patients; however, virus persistence has been documented during therapy (134, 185). With the availability of intravenous and subcutaneous preparations of immune globulin and the early recognition of this illness, fewer patients appear to be progressing to the classic description of this disease, and atypical neurologic presentations have appeared, including emotional lability, dementia, ataxia, paresthesias, deafness, memory loss, and dysarthria (162, 200). The expanded clinical spectrum of chronic EV meningoencephalitis has been revealed by PCR studies of CSF from patients with these atypical manifestations (200).

Anit-CD20 monoclonal antibody therapy with rituximab or obinutuzumab has been shown to predispose individuals to severe or fatal EV infection (201–204). Cases of myocarditis, meningoencephalitis, and hepatitis have been reported. Similarly, patients receiving chemotherapy for malignancies may also be at risk for severe EV disease (205, 206).

Acute flaccid paralysis

Although AFP has been traditionally linked to the PVs, many nonpolio EVs can also cause this syndrome. In regions of the world where the PVs have been eradicated, the nonpolio EVs and cVDPVs are now the principal causes of AFP. Several nonpolio EVs have been associated with outbreaks of AFP, including CV-A7 (207), EV-D70 (208), and EV-A71 (192). Sporadic cases of paralysis have been reported in association with isolation of an EV from the CNS or stool. In the latter situation, causality is difficult to establish due to the known prolonged fecal shedding period of the EVs. Reported nonpolio EV serotypes associated with AFP include CV-A4, -A7, -A21, -A24; CV-B2, -B3, -B5; E-3, -7, -9, -18, -19, -33; EV-D68, -A71, -B93, -D94, and -B107 and are mostly from the B species EV (50, 51, 207, 209–214). Acute flaccid paralysis due to the nonpolio EVs tends to differ from that observed with PV infection. Fever is not uniformly present at the time of onset of paralysis. It affects the upper extremities and face more frequently, is associated with a more rapid recovery, and is less likely to result in residual paralysis and atrophy. Interestingly, AFP due to the nonpolio EVs tends to be more severe in infants (209, 215).

A recent nationwide outbreak of EV-D68 respiratory disease coincided with the appearance of over 100 cases of AFP (216–219). Although EV-D68 was not isolated from the CSF in any cases, it was identified in upper respiratory tract or throat specimens of 45% of patients in one series (218), leading to presumption of its role in causality. The median age of patients was 11.5 years old (218). All had a preceding febrile illness that was accompanied by cough, nasal congestion, or a sore throat in >90%. Fever at the onset of neurologic symptoms was observed in nearly all, as were meningeal signs. Limb weakness was predominantly flaccid and asymmetric, without alterations of sensation. Unlike the AFP associated with PVs, the upper extremities were frequently involved as were the cranial nerves (VI, VII, IX, X). Pleocytosis was noted in all who had CSF drawn within 7 days of onset of neurologic symptoms. The CSF protein concentration was elevated in more than half but CSF glucose was normal in all. Magnetic resonance imaging demonstrated spinal cord abnormalities involving the anterior horns at multiple vertebral levels. Three-quarters of the cases had brainstem lesions. Neurologic sequelae were noted in all patients for whom the information was available.

Other neurologic syndromes associated with EV infections

Febrile seizures in association with RT-PCR detection of EV genome in CSF have been reported (220). Cerebellar ataxia has occasionally been associated with EV infections, as have Guillain-Barré syndrome and transverse myelitis (221, 222). All such associations suffer from the same difficulty in distinguishing pathogenicity of a throat or stool isolate from coincidental shedding.

Infections of the Neonate and Young Infant

Neonatal enteroviral infection and sepsis

The infected neonate appears to be at greatest risk for severe morbidity and mortality when signs and symptoms develop in the first days of life, consistent with either intrapartum or perinatal acquisition (66, 100, 101, 158). The timing of

maternal infection versus delivery of the infant appears to be critical; when enough time for antibody formation in the mother has elapsed, passive protection of the baby occurs. If, however, delivery occurs during maximal viremia and prior to adequate maternal antibody formation, the prognosis for the neonate is worse (66, 101, 158). An additional risk factor for disease severity may be EV serotype. A 20-year evaluation of the epidemiology of neonatal EV infections identified case fatality that ranged from approximately 17% to 20% for infants < 1 month of age infected with E-6, -11, -20, -30, and CV-B4 (109). However, only CV-B4 was associated with a higher risk of death than other EV.

The onset of clinical illness within the first 2 weeks of life is associated with a greater risk of development of severe disease (101, 158). A history of maternal illness in the form of fever, abdominal pain, or a respiratory syndrome has been reported in 59 to 68% of infected neonates (66, 100, 101, 158). Even in the youngest patients, fever is generally present. In some, hypothermia or temperature instability may occur. Nonspecific signs such as irritability, lethargy, anorexia, emesis, abdominal distention, and jaundice are common (100, 158, 223). An exanthem may be present and has been variously described as macular, maculopapular, and on occasion, papulovesicular, nodular, or bullous (Figure 5) (66). Upper respiratory findings may be present and consist of apnea, tachypnea, grunting, retractions, cough, or wheezing (100, 158, 223, 224). Diarrhea, sometimes associated with blood, may occur (223).

In the majority of neonates the infection is benign and self-limited with fever resolving in an average of 3 days and other signs and symptoms in about a week (100, 158, 223). In some, a biphasic course may occur with a mild nonspecific febrile illness preceding the onset of more severe disease (100). As the neonatal disease progresses, major systemic manifestations such as hepatic necrosis, myocarditis, and meningoencephalitis may develop (66). Severe neonatal EV disease is a multisystem organ syndrome comprised by multiple combinations of hepatitis, meningoencephalitis, myocarditis, coagulopathy, sepsis, and pneumonia. Two major clinical presentations are generally encountered: encephalomyocarditis syndrome (severe myocarditis in association with heart failure and meningoencephalitis) and hepatitis-hemorrhage syndrome (severe hepatitis with hepatic failure and disseminated intravascular coagulopathy) (109). The former syndrome is predominantly associated with infections due to CV-B serotypes, while the latter is often associated with E-11 infection (100, 101). Neurologic involvement may be variably associated with signs of meningeal inflammation, including nuchal rigidity and bulging anterior fontanelle. The CNS disease may progress to a more encephalitic picture with lethargy, seizures, and focal neurologic findings suggestive of HSV. Cardiomegaly, hepatomegaly, poor perfusion, cyanosis, congestive heart failure, and arrhythmias are indicative of myocarditis. The severe nature of the hepatitis is evidenced by hepatomegaly, jaundice, increased serum transaminases, and hyperbilirubinemia. Disseminated intravascular coagulation and other findings of "sepsis" can occur in a patient with illness that may be indistinguishable from that due to overwhelming bacterial infection. The pneumonia may require mechanical ventilation. Renal failure, intracranial hemorrhage, adrenal hemorrhage, necrotizing enterocolitis, and inappropriate secretion of antidiuretic hormone have been reported (66).

The reported incidences of morbidity and mortality due to perinatal EV infections are not precisely known but may be as high as 80% and 10%, respectively (100, 101, 158, 223, 225). Although a more recent report indicated that fatal outcome among EV infected neonates was found to be 3.3%, neonates had higher risk of death than persons ≥ 1 month of age (11.5 vs. 2.5%) infected with EV (109). When death occurs in neonates with EV infection it is typically due to hepatic failure with Es or myocarditis with CVs.

Nonspecific febrile illnesses of infancy

It is estimated that between 10 and 15 million people in the United States annually develop minor EV infections characterized by fever and nonspecific symptoms, with or without rashes (57, 226). These illnesses are of significance mainly for other diseases that they mimic including bacterial sepsis, other viral exanthematous diseases, and HSV infections; also, their age distribution makes them of great practical concern to the clinician. EVs are a major cause of hospitalization of young infants for suspected sepsis during the summer and fall months (227). Most affected patients are younger infants (< 1 year of age) in whom differentiation of viral illness from the more alarming causes of nonspecific fevers and rashes is extremely difficult. In a recent study, EVs were detected in the blood of 16% of infants < 36 months of age evaluated for fever of unknown origin who underwent NAA testing (228). In a prospective study of newborn infants, as many as 13% of infants born in the summer months were infected with EVs during the first month of life; 21% of those infected were hospitalized with suspected bacterial sepsis and received antibiotics or antiviral therapy (225). During the months of seasonal prevalence, about 7/1000 live births required hospitalization for neonatal EV infection. Clinical manifestations include abrupt onset of fever, usually > 39° C, with accompanying irritability; the fever may be biphasic (229). Additional symptoms, in order of decreasing frequency, include lethargy, anorexia, diarrhea, vomiting, rash (23% of patients), and respiratory symptoms. Signs and symptoms do not differ in this age group between the Es and CVs (229). Aseptic meningitis may accompany the nonspecific symptoms of EV infection in infants and there are no clinical features that distinguish between those EV-infected infants with and without meningitis (229). The systemic, global nature of this illness results in hospitalization of many of these infants to exclude bacterial sepsis. The duration of symptomatic illness in young infants beyond the neonatal period is usually 4 to 5 days.

Respiratory Illnesses

Many EV infections are accompanied by nonspecific respiratory signs and symptoms that are usually mild in nature. The summer cold, pharyngitis, tonsillitis, and laryngotracheobronchitis (croup) have been frequently reported (229). Bronchiolitis, pneumonia, and influenza-like illness are less common (229, 230, 231). Most EV respiratory illness is benign, but symptoms may persist for many days and the resultant disruption in school and workdays may be substantial. The EVs are responsible for approximately 15% of upper respiratory infections (URIs) for which an etiology is identified (232). In a 10-year review of EV-associated respiratory illnesses, 46% of patients presented with URIs, 13% with respiratory distress/apnea, 13% with pneumonia, 12% with otitis media, and fewer with bronchiolitis, croup, and pharyngotonsillitis (232). Using NAA detection, EVs have been identified in up to 18% of children with lower respiratory tract infections and in 25% with acute wheezing (233, 234). Many EV serotypes are identified in respiratory infections, approximately equally divided among the major subgroups (51, 226). The clinical manifestations of

EV-associated upper respiratory infections (URIs), otitis media, wheezing, and pharyngotonsillitis are indistinguishable from those due to other respiratory viruses. Pneumonia due to the EVs has been associated with numerous serotypes in infants and children (235, 236), including several of the newer *Enterovirus* C types (EV-C104, -C109, -C117) (237, 238). The clinical manifestations include fever, hyperpnea, and cyanosis. The laboratory findings usually include a normal leukocyte count, although extreme leukocytosis is occasionally encountered. Chest X-ray may reveal perihilar infiltrates. Fatalities have occurred in infants and young children. Histopathologic study of the lungs reveals thickening and infiltration of the alveolar septa but no necrosis or giant cells.

EV-D68 has recently emerged as a significant cause of respiratory disease. Originally identified in 1962 from four children with pneumonia and bronchiolitis (239), in the last 5 years it has been associated with clusters of respiratory infections or epidemics (236, 238, 240–243). Phylogenetic analysis of isolates obtained worldwide over the past 20 years indicates that multiple clades have emerged and spread rapidly (243), possibly accounting for the increase in reported clusters and epidemics worldwide. Because EV-D68 is acid labile and replicates poorly at 37° C, characteristics commonly found among the rhinoviruses, it is phenotypically anomalous among the EV species A-D (50, 245–247).

Clinically significant lower respiratory tract disease occurs primarily in young children and infants (236, 240–243, 248, 249). Some studies have reported asthma or wheezing as pre-existing conditions in approximately 70% to 80% of cases (240, 243). Pneumonia, bronchiolitis, asthmatic bronchitis, asthma exacerbation, and wheezing have been reported with EV-D68 infection (236, 240, 242, 243, 248, 249). Fever is seen only in approximately one-quarter to one-half of children. Hypoxia is very common. Chest radiographs may show infiltrates and atelectasis. The severity of the illness may require admission to an intensive care unit and assisted ventilation or extracorporeal membrane oxygenation. Deaths have been reported.

Syndromes Involving the Mucous Membranes and Integumentary System

Herpangina

CV-As are the most common causes of herpangina, but the syndrome has been reported with the CV-Bs, Es, and numbered EVs as well (250, 165). The highest incidence is among children 1 to 7 years old (172), but has also been described in neonates and adults. Onset is usually abrupt with high fever associated with sore throat, dysphagia, sialorrhea, and malaise. One-fourth of patients may have vomiting and abdominal pain. Early in the illness an oral exanthem appears as grayish-white vesicles measuring 1 to 4 mm in diameter. The lesions are located primarily on anterior pillars of the tonsillar fauces but may involve the posterior portion of the palate, uvula, and occasionally the oropharynx (Figure 6). The vesicles are discrete, surrounded by erythema, and usually number fewer than 20. Over 2 to 3 days the vesicles usually rupture, leaving punched-out ulcers that may enlarge slightly, while new vesicles may appear. There may also be mild cervical adenopathy, headache, myalgia, arthralgia, and, rarely, parotitis or aseptic meningitis. Clinical laboratory studies are usually normal. The fever lasts 1 to 4 days; local and systemic symptoms begin to improve in 4 to 5 days, and recovery is usually complete within 7 to 10 days of onset (172, 250, 251).

FIGURE 5 Newborn with overwhelming sepsis due to echovirus 11. Note typical enteroviral exanthem. This child's illness began within the first week of life and ended with her death due to hepatic failure at approximately 1 month of age.

Hand-foot-and-mouth disease

Although HFMD is one of the more common and unique syndromes typically associated with CV-A16, multiple other CV and EV serotypes (in particular EV-A71 and CV-A6) may be isolated (165). In outbreaks, the highest attack rates are among children <4 years of age but adults are also frequently affected (252, 253). The disease is usually mild and the onset is associated with a sore throat with or without a low-grade fever. Scattered vesicular lesions occur randomly on the oral structures, the pharynx, and the lips; these ulcerate readily, leaving shallow lesions with red areolae. Approximately 85% of patients also develop sparse grayish vesicles (3 to 5 mm in diameter, surrounded by erythematous areolae) on the dorsum of the fingers, particularly in periungual areas, and on the margins of heels (Figure 7). Occasionally, palmar, plantar, and groin lesions may appear, particularly in young children. Resolution is usually complete within 1 week (172). Neurologic complications have been associated with HFMD due to EV-A71 (165).

An atypical, more severe presentation of HFMD associated with a novel CV-A6 genotype has been observed since

FIGURE 6 Herpangina due to Coxsackie A viruses. Small, discrete vesicles surrounded by erythema are seen on the palate, uvula, and elsewhere in the posterior oropharynx.

FIGURE 7 Characteristic lesions of enteroviral hand-foot-mouth disease involving the dorsum of the hand and fingers

2008. The syndrome is associated with higher fever and a wider distribution of lesions involving the extremities, face, lips and perioral area, buttocks, groin, and perineum. The lesions tend to be vesiculobullous in character and can form large bullae. Skin erosions, ulcerations, and eschar formation are commonly seen. Individuals with active or dormant eczema may develop lesions concentrated in these areas known as "eczema coxsackium." The duration of illness is longer (mean 12 days) than with "typical" HFMD. Palmar and plantar desquamation or nail dystrophies (transverse ridges of the nail plate [Beau lines], shedding of the nail [onychomadesis]) may be observed 1 to 3 weeks and 1 to 2 months, respectively, after the illness (254–258).

Hemorrhagic Conjunctivitis

Acute hemorrhagic conjunctivitis (AHC) is associated with EV-D70 and CV-A24 variant (CV-A24v) and have both been associated with pandemics of AHC (110). Epidemics and pandemics of EV-D70 AHC first arose in 1969. Molecular fingerprinting has indicated that the common origin for all EV-D70 strains was West Africa approximately 2 to 3 years prior to the first known pandemic. Recently, enterovirus D70 was responsible for an outbreak in Florida (259).

Outbreaks of CV-A24v have also been widespread (110). Although originally confined to Southeast Asia and the Indian subcontinent, in 1985 it spread to Japan, Taiwan, Oceania, South America, and Africa. Phylogenetic analysis of CV-A24v isolates from outbreaks in Japan, Taiwan, and China indicate that the AHC outbreaks in these countries were the result of three successive waves of genetically distinct CV-A24v strains. Outbreaks continue into the present. A 2012 outbreak affected several islands in the Indian Ocean including the Union of Comoros, Mayotte, Madagascar, and Mauritius, with spread to France (260, 261).

The illnesses caused by the two serotypes are indistinguishable from each other, although CV-A24v-associated ACH cases may be more commonly accompanied by upper respiratory and systemic symptoms and may have less severe conjunctival hemorrhage (262). After an incubation period of about 1 to 2 days, rapid onset of swelling of the eyelids with congestion, lacrimation, and severe ocular pain occurs. Photophobia and blurring of vision are common. Subconjunctival hemorrhages vary from petechiae to large blotches. Epithelial keratitis is common, transient, and seldom followed by subepithelial opacities. Fever is an uncommon accompaniment. Preauricular adenopathy is frequent. Occasionally, a mucopurulent discharge from the eyes is found. The illness is generally nonsystemic, although transient lumbar radiculomyelopathy and a poliomyelitis-like illness was described in some cases (208, 263). Recovery is usually complete within 1 to 2 weeks of onset. High secondary attack rates within households are common.

Other EV serotypes have been known to cause outbreaks of keratoconjunctivitis, usually without hemorrhagic manifestations (264).

Muscular Syndromes

Pleurodynia

Pleurodynia is a misnomer for a clinical condition that is, in actuality, primarily a disease of muscle with clinical manifestations that suggest a pleuritic origin. The disease was fully characterized in 1934 by Sylvest, who also provided its geographically linked eponym, Bornholm disease (265). It is known by other descriptive designations: epidemic myalgia, devil's grip, etc. Various CV-B serotypes are the usual causes of sporadic and epidemic pleurodynia. However, it may also be caused by other EV serotypes (266).

An incubation period of approximately 4 days precedes the onset of illness, which is abrupt in about three-fourths of patients; the remainder first develop prodromal symptoms of headache, malaise, anorexia, and vague myalgia lasting 1 to 10 days. The major symptom is severe paroxysmal pain referred to the lower ribs or the sternum (51, 266, 267). Deep breathing, coughing, sneezing, or other movement accentuates the pain, which is described as knife-like stabbing, smothering, or catching; it may radiate to the shoulders, neck, or scapula and is characteristically absent between paroxysms. Abdominal pain occurs concomitantly in about half of patients but may occur alone. Abdominal pain may be more commonly seen in children (268). Other symptoms include fever, headache, cough, anorexia, nausea, vomiting, and diarrhea. Fever is usually about 38° C but ranges to 40° C and may be biphasic. The mean duration of the illness is 3.5 days, varying from 1 to 14 days. Muscle tenderness is ordinarily not prominent, nor is frank myositis or muscle swelling, but some patients experience marked cutaneous hyperesthesia over the affected areas. A pleural friction rub may be heard in 25% of patients. There may be splinting and tenderness on abdominal examination especially in the upper quadrants and periumbilical area. The chest X-ray is typically normal.

Inflammatory Myositis

It is known that EVs can cause inflammatory muscle disease because about 50% of agammaglobulinemia patients with chronic EV CNS infections also develop myositis (134). In these patients, cultivable virus is recovered from muscle tissue. An occasional patient with myositis and normal immunoglobulin concentrations has responded to gammaglobulin therapy, with improvement or resolution of the disease.

An autoimmune response to a triggering EV infection is thought to be a possible mechanism in polymyositis and dermatomyositis. Evidence for associating these rheumatologic diseases with EVs include serologic studies (269), visualization of EV-like particles by electron microscopy (270), as well as in situ and dot blot hybridization and NAA studies suggesting the presence of EV RNA in muscle tissue of these patients (271, 272). However, other investigators failed to find evidence of EV genome in affected muscles from myositis patients.

Cardiovascular Illnesses

Acute Myocarditis

The EVs are the second most commonly identified etiologies of myocarditis, although most cases of that disease may be undiagnosed or, if diagnosed, have no identifiable cause. EVs may cause between 25% to 35% of cases of myocarditis for which a cause is found based on serologic, nucleic acid hybridization, and PCR-based studies of endomyocardial biopsies and autopsy specimens (104, 273). Conversely, it is estimated that only 1% to 2% of all symptomatic EV infections have associated signs or symptoms of myocardial involvement (51), with the latter more common during CV-B infections than with other serotypes. Bias in the estimation of frequency of myocarditis during EV infection is possible in both directions; the subclinical nature of some cardiac involvement may result in an underestimate of the true cardiopathogenicity of the EVs, but the study of patients presenting with EV infections probably selects for the sickest patients and may overestimate the cardiac impact of the EVs.

Neonates and young infants (< 6 months of age) are particularly susceptible to CV-B-associated myocarditis accompanying systemic infection with those serotypes (100). Most cases occur in young adults between the ages of 20 to 39 years. Males are more affected than females (male:female ratio approximately 1.5:1). Rigorous exercise is anecdotally reported as a precedent to many cases of myocarditis; in animal models, exercise increases the incidence and severity of myocarditis during EV infections (108).

Clinical manifestations reflect the regions and extent of the cardiac involvement. Symptoms include palpitations and chest pain, often with accompanying fever or a history of recent viral respiratory illness. Arrhythmias and sudden death reflect a prominent involvement of the conduction system, which may be of very recent onset; congestive heart failure or myocardial infarction-like presentation suggest more significant necrosis of myocytes and likely longer-standing disease. Pericardial friction rub indicates myopericarditis. Electrocardiographic findings include an evolution from early stage S-T segment elevation and T-wave inversion to intermediate stage normalization to late stage recurrence of T-wave inversion (104). Myocardial enzyme elevations are detected in the blood. Magnetic resonance imaging and nuclear imaging may be of ancillary help in establishing a diagnosis of myocarditis, but endomyocardial biopsy remains the "criterion standard" technique for confirming the histopathologic diagnosis of myocarditis (104).

While most patients recover uneventfully from clinically apparent myocarditis, many have residual electrocardiographic or echocardiographic abnormalities for months to years. Smaller percentages of patients develop congestive heart failure, chronic myocarditis, or dilated cardiomyopathy.

Chronic Dilated Cardiomyopathy

Evidence suggests that some cases of acute myocarditis progress to chronic dilated cardiomyopathy. The latter syndrome is characterized by dilation and dysfunction of the cardiac ventricles. The incidence of this chronic illness is estimated at between 1 to 10/100,000 population, and the contribution of preceding EV myocarditis to those figures is highly disputed. Some serologic, nucleic acid hybridization, and NAA studies suggest ongoing EV involvement in 15% to 30% of cases, less than that in acute myocarditis but still substantial (104, 273). Other studies fail to find evidence of persistent EVs in patients with chronic cardiomyopathy (274–277).

Like acute myocarditis, heart failure, chest pain, or arrhythmias may herald the onset of recognizable disease in patients with dilated cardiomyopathy. Ventricular dilation and its concomitant physical findings of mitral insufficiency, cardiomegaly, and congestive failure dominate the physical examination, electrocardiographic and echocardiographic findings.

Gastrointestinal Illnesses

EVs derive their name from their site of replication and shedding in the GI tract; however, enteric illness (vomiting and diarrhea) is usually a minor manifestation of EV infections. Neither CVs nor Es have been epidemiologically implicated as important primary causes of acute gastroenteritis. Isolation of an EV from the feces of a patient with gastroenteritis must be interpreted with caution because it may represent asymptomatic shedding in a patient made ill by a noncultivable agent. The CV-Bs have been rarely associated with acute abdominal pain and mesenteric adenitis syndromes, which may mimic acute appendicitis. EVs have been associated with hepatitis in neonates (discussed above) and older children and on rare occasions in adults (206, 278, 279).

Possible Enterovirus-Associated Diseases

Type 1 Diabetes Mellitus (T1DM)

For nearly 50 years investigators have sought to establish a causal link between EV infection and the development of T1DM (280, 281). Fifteen EV serotypes have been reported to be associated with T1DM, all but one from the B species, and most heavily favoring the CV-Bs (282). Diabetes has been shown to occur with an increased incidence in the months following EV season, consistent with a postinfectious autoimmune disease mechanism (79). An increased incidence of T1DM has been demonstrated following community outbreaks of CV-B5 (283).

Although multiple serologic studies found higher antibody titers to CV-Bs in children with T1DM than in controls (284), others failed to do so (285, 286). A review of 26 case-controlled studies failed to provide evidence in support of or contrary to an association between CV-B infection and T1DM (287).

Occasional patients have developed anti-islet cell antibodies in proximity to acute EV infection (288). However, a nested matched case-control study of incident cases of beta-cell autoimmunity within two prospective cohorts of genetically high-risk children found no evidence that EV infections were a risk factor for the development of beta-cell autoimmunity (289).

An occasional diabetic patient has had CV isolated from the pancreas and elsewhere at autopsy (290). With the development of modern molecular techniques, investigators have searched for the presence of the EV genome or capsid protein (VP1) in serum, plasma, peripheral blood mononuclear cells, pancreatic tissue, and GI tissue of prediabetic and diabetic patients. A meta-analysis of 26 reports published from 1994 to 2010 demonstrated a clinically significant association between EV infection and T1DM (281). However, the authors noted that larger prospective studies were needed to clearly establish a temporal relation between EV infection and the development of T1DM.

A 2015 report supports the possibility of EV persistence in T1DM (291). Six recently diagnosed adults with T1DM who underwent pancreatic biopsy 3 to 9 weeks after diagnosis were studied. EV genome was detected in the medium

of cultured islet cells by RT-PCR in four patients. The EV VP1 was detected immunohistochemically in the pancreatic islets of all patients. However, only 1.7% of islets contained intensely staining VP1 cells. While promising, the report contained several weaknesses. The antibody used to detect VP1 was known to cross-react with tissue proteins. EV genome was detected in only one patient using biopsy tissue. The authors were not able to identify the EV serotype(s) responsible due to low viral titer.

Laboratory Diagnosis

Nucleic Acid Amplification

The most significant development in rapid, direct detection of the EVs has been the development of NAA tests (e.g., PCR and NASBA) (22, 23). NAA tests have become the standard for the clinical detection of the EVs. RT-PCR-based EV detection has provided a sensitive, specific, rapid, versatile, and clinically useful (22, 292, 293) method for the detection of the EVs. They have been employed for universal detection of all EV serotypes; species-, serotype-, and strain-specific EV detection; and strain-specific detection within a single serotype (22, 23, 294–299). For general diagnostic purposes universal detection of the EV is the most useful. Species- and strain-specific approaches to RT-PCR diagnosis may be useful for the screening of uncharacterized EV isolates, discrimination of wild-type PVs from nonpolio EV isolates or from vaccine strains, and for the detection of specific serotypes such as EV 71 (294, 295, 297, 298).

The first reports of sets of primers and probes for universal amplification of the EVs appeared in 1989 and 1990; all were broadly reactive among many EV serotypes and had high specificity for the EVs (300–302). All are directed at highly conserved regions of the EV 5′NTR (22). The sensitivity and specificity of RT-PCR for the detection of the EVs in CSF compared to the traditional criterion standard of cell culture has been shown to range form 86% to 100% and from 92% to 100%, respectively (23, 303). The sensitivity and specificity of EV detection using RT-PCR in serum is similarly high ranging from 81% to 92% and 98% to 100%, respectively (22). Commercial, FDA-approved assays are available for the clinical detection of the EV from CSF and have similar sensitivity and specificity to in-house developed tests (304–306).

No cross-amplification has been found with myriad nonrelated viruses, bacteria, fungi, and yeast (22). This being said, some primer pairings may amplify a limited number of rhinoviruses (307), a finding that is not surprising given that the rhinoviruses are members of the same genus (4, 3) and share 5′NTR sequence motifs. This should be of minor importance when samples such as CSF, serum, or nonrespiratory tract tissues are being tested. However, for commercially available multiplex NAA assays for the detection of respiratory pathogens that lack the ability to differentiate between EV and rhinoviruses, this may be problematic.

In the clinical setting RT-PCR is extremely sensitive for the detection of EV in CSF specimens. Paired analysis (i.e., cell culture and RT-PCR testing) of RT-PCR detection of EV from CSF specimens demonstrated it to be consistently and substantially more sensitive than cell culture (22). Using RT-PCR, it is possible to detect EV CNS infection in agammaglobulinemic patients with associated meningoencephalitis or encephalopathy in the absence of cell culture recovery of viruses (23, 200). RT-PCR results can be available in hours, a substantial time saving when compared with detection by cell culture. This reduction in time to positive detection has been documented to shorten hospital stays, reduce antibiotic use, and affect patient management (292, 293, 308). Testing of CSF for EVs should not be determined by the presence or absence of pleocytosis. Multiple studies have documented a high proportion of young infants with EV CNS infection but without pleocytosis (169, 170).

Virus Isolation

Dr. John Enders and colleagues received the Nobel Prize in 1954 for successfully propagating PV in a cell culture system (309). Isolation of EVs in cell culture and recognition of cytopathic effect require a high level of expertise and are labor intensive. Some serotypes, particularly within the CV-A, do not grow in cell culture (310, 311). Of greater significance, 25% to 35% of specimens from patients with characteristic EV infections will be negative by cell culture (312) because of antibody neutralization; inadequate collection, handling, and processing of the samples; or intrinsic insensitivity to the cell lines used. EVs that do grow in cell culture may do so slowly. Mean isolation times for EVs from CSF range from 3.7 to 8.2 days (313); EVs from other sites, where viral titers are higher, often grow more rapidly (312, 314, 315). Shell vial culture may shorten detection time to 2 to 3 days.

For reasons listed previously, isolation of EVs by cell culture has been replaced by NAA testing as the method by which the majority of clinical laboratories attempt detection of the EVs. Monkey kidney cell lines have good sensitivity for PV, CV-B, and E. RD cells and HuT 292 cells, derived from a human rhabdomyosarcoma and pooled human saliva, respectively, are the most sensitive for detection of CV-As (310). Even with the addition of newer cell lines as well as those genetically engineered to express the PV receptor or decay-accelerating factor, L20B and BGMK-bDAF, respectively, no single cell line is optimal for all EV serotypes (23). Most laboratories use a combination of a primary monkey kidney cell line with a human diploid fibroblast line. Commercially available mixtures of cell lines may be useful in attempting to achieve maximum sensitivity (23). Cell culture continues to be important in the detection and surveillance efforts by the World Health Organization for control and surveillance of wild-type PV strains and VDPVs (290).

Although isolation using suckling mice inoculation is the most sensitive method for laboratory diagnosis of CV-A infections (310), it is rarely performed any longer because of the difficulty of the technique and need for animal maintenance.

Serologic Assays

Serologic testing has a limited role, if any, in EV diagnosis because of the great diversity of EV serotypes and the failure to identify a single common EV antigen, lack of sensitivity, and cross-reaction with non-EV members of the *Picornaviridae* family. The sensitivities of hetero- and homotypic immunoassays for the diagnosis of EV infections pale in comparison to that of RT-PCR, ranging from 34% to 75% in patients with RT-PCR-confirmed EV meningitis and 46% in patients in whom EV was detected in stool by RT-PCR (271, 316).

If the specific serotype of an infecting EV is known or suspected, confirmatory homotypic serology can be performed on individual patients to document a rise in antibody titer from the acute to the convalescent phase of infection, thus providing useful epidemiologic information. In a situation where an EV is recovered from feces or throat of a

patient with unusual clinical manifestations, the etiologic role of the EV may be more firmly established by documenting a four-fold rise in antibody titer to that serotype in paired acute and convalescent sera. In the usual scenario when a patient presents with meningitis or other acute manifestations of illness and an EV is suspected, serology is not a practical option. A recent report further supports this, concluding that virus serology had no relevance for the diagnosis of myocarditis (317).

Specific Identification of the EVs

The traditional method for determination of EV serotype is the use of intersecting pools of equine antisera, as established by Lim and Benyesh-Melnick (LBM pools) (318–320). Two sets of antibody pools are available, eight pools (A to H) of antisera that identify 42 EV and seven pools (J to P) of antisera that identify 19 strains of CV-As. A checkerboard analysis localizes the isolate to a single serotype designation on the basis of the pattern of neutralization with the intersecting serum pools. The LBM pools are available in limited supplies from the World Health Organization.

Limitations exist with this methodology (321, 322). Principally, the LBM pools fail to identify the newly identified EV. Because the LBM pools were developed over 50 years ago, genetic drift has given rise to antigenic variants of the prototypic strains that may be difficult to conclusively serotype. Additionally, viral aggregation may negatively affect the performance of the pools, the procedure is labor intensive and time consuming, and the supply of LBM pools is limited.

Broadly reactive and serotype-specific EV monoclonal antibodies in conjunction with cell culture have been used. However, this approach should best be used as a preliminary screen for species or serotype identification due to lack of sensitivity and cross-reactivity with the rhinoviruses (323).

Nucleic acid amplification technology has provided the means to "molecularly type" the EV (13, 14, 324, 325). This approach relies on amplification of the sequence of the EV VP1 coding region. The amplicons generated are rapidly sequenced using PCR and computationally compared to a database of known EV VP1 sequences to establish identity. This method greatly reduces the time required for identification of the EV from weeks to days. "Molecular typing" has been extremely useful in the identification of isolates considered "non-typeable" EVs due to a failure to be neutralized by the LBM pools (321). This approach has been used to successfully identify new EV types (321, 326–328). It is possible to perform species identification directly from clinical specimens (324).

The determination of the specific serotype of infecting EVs is generally unnecessary because the diseases caused by the EVs are not serotype specific. Further identification to the level of specific serotype may be useful under certain circumstances such as epidemiologic studies of patterns of EV infections, descriptions of unusual or novel clinical manifestations, and incursions of rarely encountered serotypes. In most circumstances it is adequate for the diagnostic laboratory to report the presence of "an EV" without further detail.

A notable exception to this principle is where distinguishing between wild-type PVs, vaccine strain PVs, VDPV, and nonpolio EVs is critical to interpretation of viral culture results. These include regions of the world where live attenuated PV vaccines continue to be used and where surveillance for wild-type PVs and VDPV is undertaken as part of the global effort for eradication of polio (299). The standard method for distinguishing between PVs and nonpolio EVs employs neutralization of the isolate with a pool of antisera directed against the three PV serotypes (299). Molecular typing of wild-type, vaccine, and vaccine-derived PVs is extremely useful (297–299).

Interpretation of Results

The body site at which EVs are isolated is critical to the interpretation of EV detection assays and to the differentiation between EV "shedding" and actual EV-associated disease. The nasopharynx and GI tract are permissive sites of infection, i.e., EVs have ready access to these sites and may infect and subsequently be "shed" from them for weeks to months. Detection of EVs by virus isolation or NAA at these sites must be interpreted cautiously because their presence alone does not establish causality of the illness in question (329). Indeed, virtually 100% of patients with EV aseptic meningitis will have detectable EV in feces (315), but most persons shedding EV in the feces at any particular time are asymptomatic; feces are thus the most sensitive and least specific site for detecting true EV-associated illness. Since the shedding period in the nasopharynx after EV infection is shorter than in the feces, the specificity of an EV isolate from the nasopharynx for true causation of current symptoms is better than with feces but far short of a definitive association. In contrast, the CNS, bloodstream, and genitourinary tract are not usually infected with EVs, i.e., detection of virus in specimens from these sites implies true invasive infection and a high likelihood of association with current illness.

A notable exception to the above relates to the diagnosis of neonatal EV infections occurring within the first 2 weeks of life. Because the incubation period for the EV is 3 to 6 days, the identification of EV in the stool or oropharynx of newborns with EV-compatible syndromes is highly supportive of their role in disease.

Rare reports of co-infections of the CSF by bacteria (*S. pneumonia*, *H. influenzae*, *S. agalactiae*, *N. meningitidis*), mycobacteria (*M. tuberculosis*), or viruses (St. Louis encephalitis virus, HSV) and EVs have appeared (187, 330–335). In cases of bacterial or mycobacterial co-infection, the bacteria/mycobacteria-associated clinical signs and symptoms usually dominate. In the much more common situation, where the clinical presentation is typical of viral meningitis, coinfection with a clinically "silent" bacteria would be extraordinarily unlikely. Hence, identification of an EV from a site not ordinarily infected in a patient with a clinically compatible illness is usually sufficient evidence for establishing EV causality.

PREVENTION

The provision of potable water and adequate sanitation are cornerstones in public health efforts toward the prevention of EV infections. Handwashing has been shown to be effective in the prevention of EV infections (336). Vaccines are available only for the PVs, and those provide no protection against the nonpolio EV serotypes. In an attempt to eliminate the few remaining vaccine-associated cases of poliomyelitis that occurred annually in the United States, the use of live attenuated oral PV vaccines was abandoned nearly two decades ago. Today, only inactivated PV vaccines are used for immunization of children in this country. Elsewhere in the world, however, the majority of countries continue to use OPVs as a part of their vaccination regimens.

Because of the burden and severity of EV-A71 disease in China, promising efforts are under way to develop vaccines against the most common subgenotype, C4, circulating in

that part of the world. Three candidate inactivated EV-A71 subgenotype C4 vaccines have undergone successful Phase III studies (337–339). All were safe and well tolerated. Seroconversion, as defined by a 4-fold increase in baseline neutralizing titer, occurred in 88.1%-100%. Efficacy against HFMD and other EV-A71 related disease ranged from 90% to 97.4% and 80.4% to 88%, respectively. However, antibody titers waned after the first 6 months, indicating that boosting immunity may be necessary. At least two of the vaccines cross-neutralized several other EV-A71 genotypes and subgenotypes, suggesting that they could induce cross-protection against other strains (340).

TREATMENT

As with other viral pathogens, there are several steps in the replication cycle of the picornaviruses that are potential targets in antiviral therapy. Cell susceptibility, viral attachment, viral uncoating, viral RNA replication, and viral protein synthesis have all been studied as targets of antipicornaviral compounds.

Immunoglobulin

The primary mechanism of clearance of EVs by the host is humoral immunity. As noted above, patients who lack antibodies because of congenital or acquired immunodeficiencies are uniquely susceptible to infections with the EVs (134). Similarly, normal neonates are at high risk for severe EV disease because of a relative deficiency of EV antibodies (158). Antibodies act by binding to EVs and preventing attachment and binding to host cells.

Immune serum globulin has been used prophylactically and therapeutically against the EVs in two situations, the neonate and the immunocompromised host. Neonates may develop an overwhelming sepsis syndrome from transplacental/peripartum acquisition of EV infection. The high mortality of this disease, coupled with the known association of severe EV disease with absolute or relative antibody-deficiency states, has prompted numerous investigators to administer antibody preparations to neonates with EV sepsis. Anecdotal reports of clinical success with maternal serum or plasma (341) or commercial immunoglobulin preparations against a variety of EV serotypes causing neonatal sepsis have been reported; other reports describe progressive disease and death despite such therapy (342, 343). A blinded, randomized controlled study was too small to demonstrate clinical benefit but did show a reduction in viral titer in infants receiving intravenous immunoglobulin (IVIG) preparations that were subsequently shown to contain high antibody titers to the infecting serotype. Individuals with congenital or acquired antibody deficiencies are also at risk for severe EV infections. Since antibody-deficient patients have begun receiving maintenance supplementation with IVIG, the incidence of chronic, progressive EV meningoencephalitis has fallen, demonstrating the prophylactic benefit of these preparations, and the clinical profile of patients developing such infections has been modified (134, 162). Therapeutic efficacy in established EV meningoencephalitis in antibody-deficient patients has only been anecdotally studied.

Capsid-Inhibiting Compounds

Capsid-inhibiting compounds have been the most extensively studied antiviral agents for the therapy of the EV. Capsid-inhibiting compounds block viral uncoating and/or viral attachment to host cell receptors. As noted above, the resolved 3-dimensional structure of the EVs reveals a "canyon" formed by the junctions of VP1 and VP3. Beneath the canyon lies a "pore," which leads to a hydrophobic pocket into which a variety of diverse hydrophobic compounds can integrate. Although the compounds integrate into a virus capsid via a number of noncovalent, hydrophobic-type interactions, the affinity is high. Several hypotheses have been proposed for the mechanism of picornavirus inhibition by compounds that affect the function of the virus capsid. Filling the hydrophobic pocket results in increased stability of the virus, making the virus more resistant to uncoating. The increased stability of the virus-compound complex is evidenced by the resistance to thermal inactivation (344). It is also possible that a degree of capsid flexibility may be required for uncoating, and activity of these compounds within the hydrophobic pocket may reduce this necessary flexibility, inducing a more rigid structure. Alternatively, changes in the conformation of the canyon floor as a result of drug activity within the underlying pocket may affect the attachment of the virus to the host cell receptor (345). It has been shown, however, that such perturbations in the canyon floor do not absolutely correlate with antiviral potency (346).

Two drugs, pleconaril and pocapavir, have undergone clinical trials in humans. Pleconaril demonstrates broad spectrum and potent anti-EV activity and is highly orally bioavailable, approaching 70% in humans (347–349). Published reports of the therapeutic efficacy of pleconaril provide a mixed picture. A challenge study of the efficacy of pleconaril in the reduction of viral shedding, relief of symptoms, and decrease in severity of clinical illness following CV-A21 infection in nonimmune individuals demonstrated a statistically significant decrease in nasal secretion viral titers in subjects compared with controls (350). The mean volume of nasal secretions in pleconaril treated subjects was consistently less than in controls. Mean respiratory symptom scores in placebo-treated individuals were significantly higher than in the treated group.

A clinical trial of pleconaril for the treatment of EV meningitis in infants (102) demonstrated no significant difference between the treatment and placebo groups with regard to EV detection by culture or PCR, duration of hospitalization, or symptoms. It was concluded that the small numbers of subjects, low yields of serial viral cultures, and the short, benign clinical course precluded the ability to demonstrate efficacy.

A post hoc subgroup analysis of two efficacy trials that failed to define clinical benefit for FDA registration of pleconaril indicated that it was beneficial in the acceleration of headache resolution (103). Pleconaril has been used in a compassionate release protocol for the treatment of more than 90 patients with potentially life-threatening EV infections, 38 of whom were followed long enough to assess therapeutic responses (351). Seventy-eight percent of patients had a favorable clinical response, while 92%, 88%, and 60%, respectively, with virologic, laboratory, or radiologic abnormalities responded favorably.

A prophylactic study for the prevention of picornaviral respiratory tract infection demonstrated an increase in menstrual irregularities in women receiving pleconaril (349). Investigation revealed induction of cytochrome P-450 3A activity by pleconaril and raised the possibility of the potential for drug interactions, in particular, interference with oral contraceptives and antiretroviral drugs. The FDA concluded that the risks of pleconaril use outweighed its modest benefits and did not license pleconaril for the treatment of the common cold (352).

A recently published study of pleconaril therapy of neonates with suspected EV sepsis found that in the treatment group, EV-infected subjects became culture negative from all sites combined faster than subjects in the placebo group. Fewer subjects in the treatment group remained PCR-positive from the oropharynx when last sampled. Intent-to-treat analysis demonstrated a reduction in mortality in the treatment group. The authors concluded that the results supported potential efficacy and that they warranted further evaluation of pleconaril for the therapy of neonatal EV sepsis (353).

The Committee on Development of a Polio Antiviral and Its Potential Role in Global Poliomyelitis Eradication of the National Research Council has recommended that at least one polio antiviral drug be developed to supplement efforts currently being employed for control of poliomyelitis outbreaks in the posteradication era (354). Pocapavir has been shown to be a potent inhibitor of wild-type, vaccine, vaccine-derived, and laboratory strains of PV. It has been shown to have potent and rapid antipolio activity in a randomized, double-blinded, placebo-controlled human monovalent oral PV type-1 vaccine challenge study. Primary and secondary endpoints were the time to clearance of PV from stool and total stool virus titer, respectively. Nearly half of the pocapavir recipients had clearance of PV from the stool before placebo recipients. Drug resistance was not observed in this group. The remainder of the pocapavir recipients responded similarly to placebo recipients. Pocapavir resistance was higher than anticipated in this group but was found to be due to PV transmission and reinfection within the study unit, not due to the development of resistant PV (355). A single report of the successful treatment of a non-polio EV exists (356).

Supportive care for the patient with EV meningitis is usually adequate to assure complete recovery. Attention to fluid balance is necessary to avoid or ameliorate the syndrome of inappropriate antidiuretic hormone or brain edema. Electrolytes, urine, and serum osmolality may require monitoring. Brain edema is a rare complication of EV meningitis but is readily managed with mannitol. Seizures may result from fever alone or may reflect direct viral or indirect inflammatory damage of brain parenchyma. Phenytoin or phenobarbital are the preferred agents for managing this complication. Patients with EV-A71 encephalitis or EV-D68 lower respiratory tract infection may require assisted mechanical ventilation.

Treatment for the neonate with sepsis or the child or adult with myocarditis is likewise symptomatic. Maintaining adequate blood pressure is paramount in each of those syndromes. Steroids have been widely debated in the therapy of myocarditis but are felt to be contraindicated in most cases (104). No significant benefit has been reported for other immunosuppressive classes of drugs either.

Adequate hydration is the only indicated therapy in children with herpangina and HFMD due to the EVs. Other respiratory manifestations of EV infections are managed symptomatically.

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Rhinovirus

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Rhinoviruses (RVs), members of the *Picornaviridae* family (1), constitute the largest group of respiratory viruses. RVs are recognized as causing more than 50% of all acute respiratory infections and represent the single most important causative agent of common colds. The name "rhinovirus" stems from the virus's special adaptation to infect the nasopharynx. It was already known in 1930 that "colds" were easily transmitted from human to human and to apes, and that the responsible agent was probably a virus (2, 3), but it was not until 1956 that the first RV was discovered by isolation in cell culture (4, 5). The discovery of the low temperature optimum (32–35°C) for viral replication (6), the development of sensitive primary human embryonic lung cell cultures (WI-38 and MRC-5), and the continuous H1-Hela cell line (7, 8) facilitated the isolation, classification, and epidemiological and biological studies of RVs. A total of 100 serotypes were identified over the next 30 years (9). The first complete genome sequence was determined for RV14 in 1984 (10), a reverse genetics system was developed in 1985 (11), and the X-ray crystallographic structures of the viral capsids of five serotypes (1A, 2, 3, 14, and 16) were solved soon afterward (12–17). The recent use of more sensitive RT-PCR-based molecular assays for RV identification has generated clear evidence that RV infections are also common causes of more severe lower respiratory illnesses, such as bronchiolitis, pneumonia, exacerbations of asthma, and other chronic lung diseases (18–22). RT-PCR has also led to the discovery of more than 50 genotypes of previously unrecognized RVs that belong to a new species (RV-C). These viruses escaped traditional culture-based detection (23–26). This discovery has promoted a new wave of interest in RV research.

VIROLOGY

Classification

RVs belong to the family *Picornavirdae* and were traditionally classified into their own genus, *Rhinovirus*, according to their clinical and biological characteristics (9, 27). As molecular methods became available for virus identification, RVs were re-classified and merged into the genus *Enterovirus* because of similar molecular genetic properties (1). RVs are comprised of three species: rhinovirus A, B and C (RV-A, RV-B, and RV-C). Species A and B, consist of the 100 serotypes that were identified by traditional methods and species C consists of over 50 genotypes that have been identified by molecular genetics methods.

Acid Lability and Buoyant Density in Cesium Chloride

One of the defining characteristics of the human RVs is their acid lability; their infectivity is reduced at least 10-fold when incubated at pH 4 for one hour at 37°C in 0.1 M sodium citrate. In contrast, most human enteroviruses are stable under these conditions. All human RVs so far examined exhibit higher buoyant density (1.38 to 1.42 g/ml) in cesium chloride gradient than do enteroviruses, which characteristically band at 1.34. This property is due mainly, if not entirely, to replacement of potassium ions bound to the RNA genomes of RVs by heavier cesium ions. Cesium-potassium exchange does not occur in enteroviruses because the protein shell is impermeable to the cesium ion.

Serotypes

One of the characteristic features of human RVs is the large number of serotypes. The 100 serotypes (1A, 1B to 86, and 88 to 100) were identified with culture-based, biochemical, and serological methods between 1956 and 1987 (9). The traditional method for identification of a RV serotype is neutralization of a virus inoculum containing 30 to 300 $TCID_{50}$ (tissue culture infectious dose) by an antiserum dilution containing 20 units of antibody (28). One antibody unit is the minimum amount of antiserum that neutralizes 30 to 300 $TCID_{50}$ of that virus. Each serotype was defined by 20-fold or greater differences in antisera neutralization titers during reciprocal cross-neutralization tests against the other 99 serotypes. Antigenic subgroups have been identified among 50 serotypes-(1A,1B), (2,49), (3,6,14,79), (9,32,67), (11,15,40,74,76), (12,78), (13,41), (22,61), (5,17,19,21,29,30,42,43,44,70), (18,36,50,58,89), (38,60), (39,54), (48,55), (56,57), (59,63,85), and (66,77)-in a comprehensive serological study of 90 serotypes (29). Moreover, certain serotypes, such as type 17, appear prone to serohypervariation (30).

Serovariability of RV may be related to the size of the receptor binding site (footprint) on the virion surface (31). Unlike rhinovirus, closely related poliovirus, has only three serotypes that use CD155 as their receptor. Like ICAM-1

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(the receptor for 88 rhinovirus serotypes), CD155 binds to the canyon area on the virion surface. Structural studies of virus-receptor complexes show that ICAM-1 has a smaller footprint than CD155. This could permit the emergence of more viable antibody-escape mutants because the rhinovirion surface has more amino acid residues that could be mutated without disabling virus binding to the receptor.

Phylogenetic Groups

In the past 10 years, RT-PCR-amplification of viral genomic RNA, DNA sequencing, and phylogenetics have gradually become routine procedure for the identification of RVs in clinical samples, because this approach is significantly more sensitive, faster, and easier to perform than traditional culture and serology-based identification. With phylogenetic analyses, the genome sequences of the 100 RV serotypes segregate into 2 groups: RV-A, consisting of 75 serotypes and RV-B, consisting of 25 serotypes (32–35). When molecular methods were used for RV identification, a large group of previously unculturable RVs were discovered (23–26). These viruses were detected by RT-PCR assays using RV-A and -B specific primers and their sequences were most closely related to RV-A and -B sequences in BLAST searches of the GenBank database. However, they segregated into a third group, with 30% to 40% pairwise sequence divergence from RV-A and -B, in phylogenetic trees. This new group is named RV-C (species C). RV-Cs are not newly evolved viruses because they could be found frequently in frozen clinical samples from the early 1980s, the oldest analyzed samples to date (36).

RV-C Viruses

RV-C viruses were first detected by sequencing of PCR fragments of 5′ nontranslated regions (NTR) and capsid protein coding VP4-VP2 regions of viral genomic RNA in human nasal samples (23–26). To date, over 50 types of RV-C have been tentatively identified by partial genome sequences, and 19 of them have been definitively identified with their complete genome sequences (37). RV-C does not grow in traditional cell culture systems, so their biological and immunological properties are still poorly understood. Recently, several groups have developed methods to grow them in sinus organ culture, differentiated airway epithelial cells in air-liquid interface culture, or HeLa cells transfected with the putative RV-C receptor (38–42).

Receptor Types

The 100 human RV serotypes are divided into two groups on the basis of receptor specificity (Fig. 1). The "major" receptor group, containing 88 serotypes, utilizes the intercellular adhesion molecule-1 (ICAM-1; Fig. 2A) as receptor for infecting host cells (43). This receptor is a member of the Ig superfamily and maps to human chromosome 19 (43). Adhesion molecules like the ICAMs play a natural role in allowing cells to adhere to each other or to extracellular matrix molecules. The major group RVs are highly specific for ICAM-1 from humans and apes, such as chimpanzees and gibbons. Twelve serotypes, constituting the "minor" receptor group, utilize three members (LDLR, VLDLR, and LRP) of low-density lipoprotein receptor family as receptors (44, 45). The minor group RVs can bind to LDLR of the mouse. All 12 members of minor receptor group are RV-A viruses, while major receptor group consists of 63 RV-A and all 25 RV-B viruses. The study of the receptor(s) for RV-C is still at its early stages due to the complexity of growing RV-C in culture. Recently, a putative receptor for RV-C viruses has been identified as cadherin-related family member 3 (CDHR3). HeLa cells transfected with CDHR3 expression vector support the replication of three RV-C types (C2, C15, and C42) tested (40).

Composition

The virion has no lipid envelope and is composed of a single-stranded RNA genome tightly packed into the center of a protein shell known as the capsid (Fig. 2B). The concentration of purified virions can be measured spectrophotometrically with a conversion factor of 9.4×10^{12} virions per OD_{260} unit (46). Water is an important part of the virion structure. In its fully hydrated state (observed during conventional X-ray crystallography or by cryoelectron microscopy), the particle is about 30 nm in diameter. When dried (e.g., during preparation for conventional electron microscopy), the virion shrinks about 30% and loses infectivity. The protein shell, about 5 nm thick, is composed of 60 copies of each of four viral proteins, termed VP1-VP4. Protein subunits called protomers, consisting of one copy of each of the viral proteins, are organized into 12 pentamers. Each of the 12 pentamers contains a prominent depression or "canyon" in VP1 running moat-like around a central plateau (i.e., around the 5-fold axis of symmetry) (47). Amino acid residues at the canyon base are more conserved than residues at exposed viral surfaces.

The canyons of major group RVs are the binding sites for the ICAM-1 receptors (48, 49). Cryoelectron microscopy (using purified fragments consisting of the distal two domains of ICAM-1) has shown that the canyon (Fig. 2C) is the site at which the receptor binds to RV16 and that purified virions can bind up to 60 such receptor fragments (49). Initially, it was hypothesized that the 20 Å deep canyon was used to hide the viral receptor attachment site on a viral surface from immune surveillance (50). However, the X-ray structure of the RV14-Fab complex showed that the antibody molecule penetrated deep into the canyon

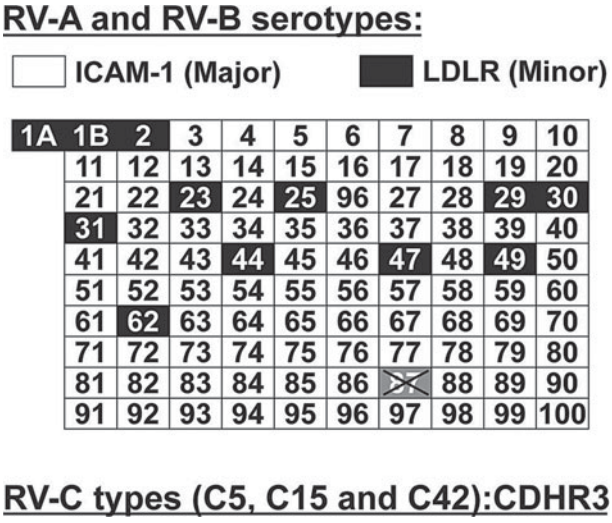

FIGURE 1 Receptor groups of rhinoviruses. Majority of RV serotypes (63 RV-A and all 25 RV-B) use ICAM-1 as the receptor. The remaining 12 RV-A serotypes bind to low-density lipoprotein receptor (LDLR) family proteins. RV87 was originally misidentified as a RV and has been reclassified as an enterovirus (EV-D68). The receptor for RV-C has been recently identified as cadherin-related family member 3 (CDHR3) protein.

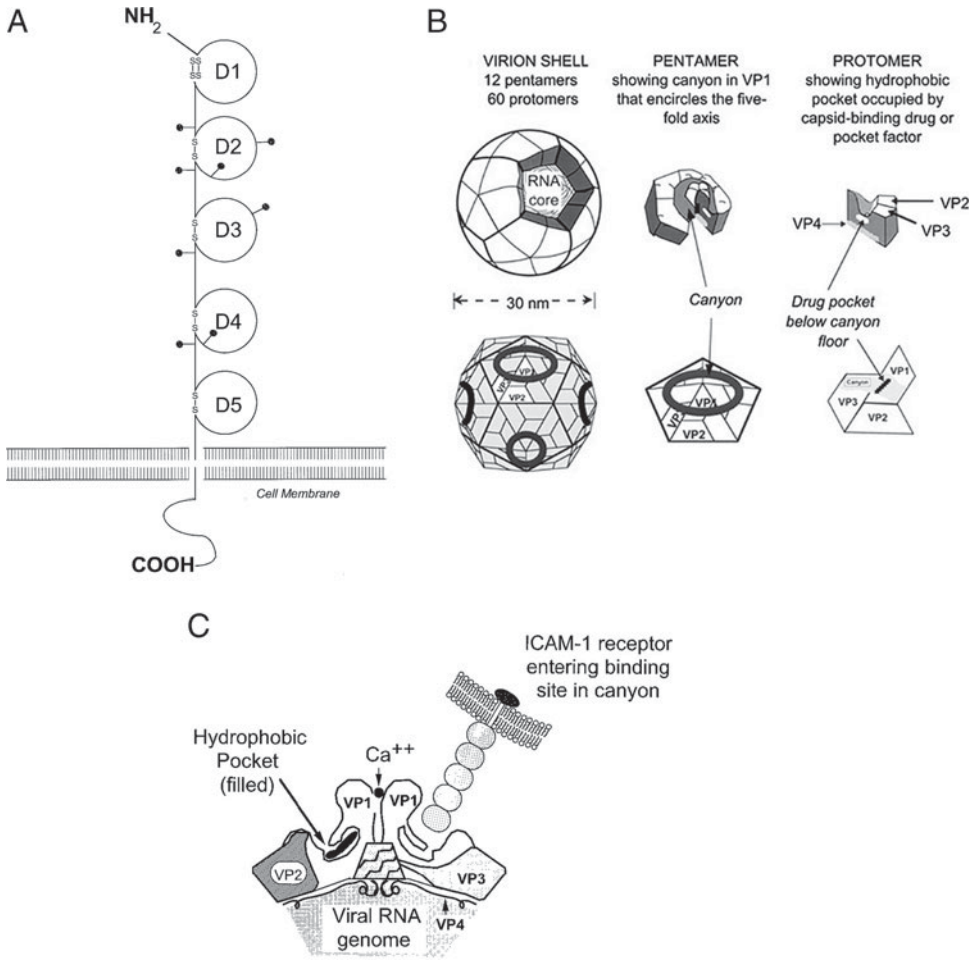

FIGURE 2 (A) Schematic drawing of ICAM-1, the receptor used by the major group RVs. S-S represents disulfide bridges, and small circles represent sites of glycosylation. (B) Key features of the structure of a human RV. The virion shell consists of 12 pentamers, one of which has been removed to show the approximate location of the RNA packed tightly into a central cavity. Each pentamer, in turn, consists of five wedge-shaped protomer subunits. The canyon (shaded) is shown encircling the five-fold axis of the pentamer; the hydrophobic (drug-binding) pocket is indicated below the floor of the canyon in VP1. (C) A side view of the pentamer showing the spatial relationship between the receptor-binding site and the hydrophobic pocket. An ion, located at each pentamer center in serotypes 1A, 14, and 16 is tentatively identified as calcium.

space (51), and so the canyon may not conceal the receptor-binding site at all. Interestingly, minor group RVs do not use the canyon for receptor binding; rather, the minor group receptor binds to the plateau area near the tip of the icosahedral five-fold vertex (52). Molecular modeling indicates that the RV-C capsid has less pronounced canyons that likely do not contain receptor binding sites (40). Nevertheless, the canyons of both major and minor group viruses play a critical role in the uncoating (release of genomic RNA) step following attachment by providing a flexible region for the conformational change in the viral capsid. It is thought that the binding of multiple receptors per virus particle (receptor "recruiting") triggers a major conformational changes of the capsid proteins, accompanied by 20% expansion of the shell, to allowing the eventual release of RNA (47).

At the base of the canyon lies a hydrophobic "pocket" which may be empty (e.g., RV14) but which in many other RVs (e.g., RV16) is filled with a still uncharacterized molecule called "pocket factor." A pocket factor may play a role in the replication cycle of RVs by regulating the stability and receptor-induced conformational changes of the viral capsid (47, 53, 54). This pocket is the binding site for several classes of antipicornavirus capsid-binding antivirals (e.g., pleconaril and vapendavir). In general, capsid-binding antivirals block the binding of major receptor RVs to ICAM-1 and inhibit uncoating of receptor-bound minor group RVs (55, 56). For RV-C, the pore to the pocket is more constricted, and these viruses are resistant to several capsid binding antivirals including pleconaril (57).

Immunoglobulins bind to the virion surface to neutralize infectivity of RV. Several mechanisms for antibody-mediated neutralization of RVs have been proposed, including aggregation, virion stabilization, induction of conformational changes, and abrogation of cellular attachment (58, 59). Taken together, numerous biochemical and structural studies suggest that neutralization of RV *in vitro* results from steric blockage of the interaction between the virus and its receptors due to the binding of the antibody molecules on the virion surface (59).

Genome

The rhinoviral genome is a single strand of positive-sense RNA about 7200 bases long. Sequence analysis indicates a genome organization similar to that of poliovirus, the prototype of enteroviruses (Fig. 3A). Both viruses contain poly (A) tracts which are required for infectivity of the viral genomic RNA, and both are covalently linked through the 5′-terminal uridylic acid to a small viral protein (VPg) which can be removed from the genomic RNA without reducing its infectivity. The viral genomic RNA contains a single open reading frame (ORF) for a large polyprotein of about 2,200 amino acids. Both polioviruses and RVs have unusually long nontranslated regions (NTR; 600 to 750 bases) at the 5′-ends with distinctive secondary and tertiary structures called internal ribosomal entry sites (IRES). IRES enable the translation of viral genomes by promoting the internal binding of the ribosomes to viral RNAs since rhinoviral RNAs lack 5′-terminal caps that are used by most cellular mRNAs for ribosome binding (47). IRES are used by many positive-stranded RNA viruses and some eukaryotic genes. The RV genome also includes a relatively short 3′-NTR that is required for efficient viral replication (60).

Proteins

The organization of the polyprotein of human RVs is also very similar to that of poliovirus (Fig. 3B), though much less is known of the details of rhinoviral processing and assembly because of the less vigorous translation of rhinoviral RNA in cultured cells and incomplete shutoff of host protein synthesis by viral proteases (54). The RV genome, like that of polio, is organized into three major regions based on proteolytic processing: P1, P2, and P3, representing genes for precursor proteins. These precursors are subsequently cleaved into 4, 3, and 4 end products, respectively. Protein P1 is a precursor for the four coat proteins. P2 and P3 are precursors for proteases 2A and 3C and for proteins required for replication of the viral RNA.

Before assembly, the coat precursor protein, P1, is cleaved twice to form mature protomers (VP0, VP1, VP3), which then package RNA to form noninfectious provirions containing 60 protomer subunits (Fig. 4) (54, 61). The maturation cleavage of VP0, producing VP4 and VP2, occurs only after the RNA has been packaged in the protein shell (54). The VP0 cleavage site lies buried inside the shell near the viral genomic RNA: thus, the protease event responsible for this cleavage is thought to be an element of the coat protein and may actually use bases in the viral RNA as part of its active site (15). The P1 region is released by a cis-cleavage at the N-terminus of the 2A protease. All remaining cleavages are carried out by the 3C protease or its precursor form, 3CD. A molecule of myristic acid, which appears to play a role during virus assembly and disassembly, is covalently linked to the amino termini of VP4 and its precursor forms.

BIOLOGY

Multiplication Strategy

Multiplication of RVs is believed to take place entirely in the cytoplasm. The initial event in infection is attachment of the virion to specific receptor units embedded in the plasma membrane (Fig. 5, step 1). Receptors then bring the virion close to the membrane. Recruitment of additional receptors (step 2) eventually triggers the uncoating of the virion that involves extrusion of the protein VP4 and delivery of the viral RNA genome across the membrane and into the cytosol (step 3) where translation can begin. The study of the

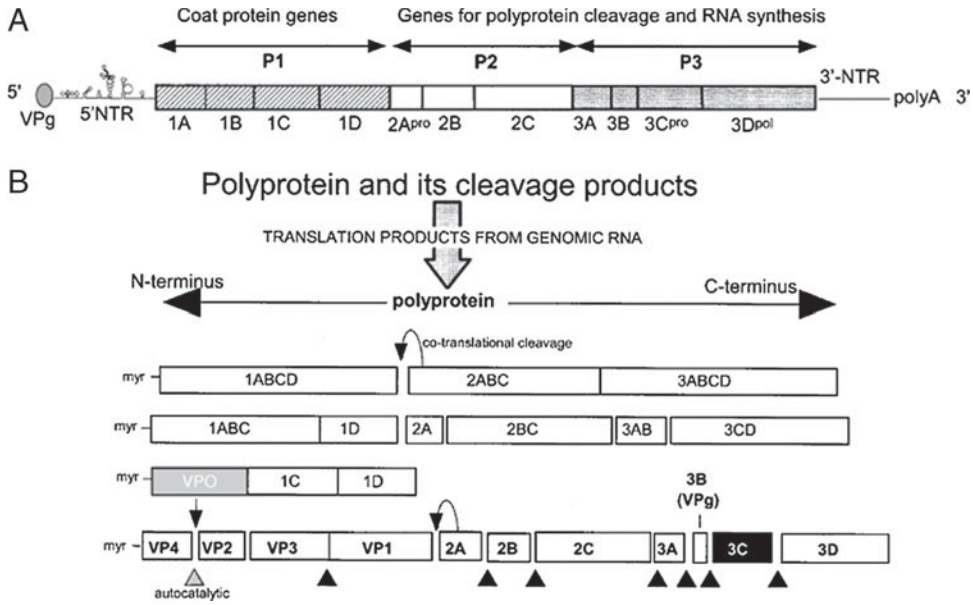

FIGURE 3 (A) RNA genome of a human RV. P1, P2, and P3 refer to precursor proteins that are subsequently processed to produce 11 end products. (B) Cleavage of the polyprotein is accomplished by two viral proteases, 2A and 3C. The 2A protease co-translationally releases the coat precursor, P1, from nascent polyprotein, whereas the 3C (or precursor 3CD) protease cleaves all the remaining precursors and intermediates except for VP0 (1A-1B). Cleavage of VP0 to VP4 (1A) and VP2 (1B), maturation cleavage, occurs only after the RNA has been packaged in the protein shell. The VP0 cleavage site lies buried inside the shell near the RNA; the active site for this cleavage is not yet precisely known and might include bases in the RNA genome. The amino termini of coat proteins P1 and VP4 are blocked by a myristoyl group. Cis-cleavage of the N-terminus of 2A is shown by an arrow; black triangles indicate 3C cleavage sites.

uncoating pathway of RVs has been difficult because the interpretation of biochemical data is clouded by the fact that only 1% or fewer input virions initiate a successful infection. One major reason for the low infection efficiency is elution of noninfectious 135S and 80S subparticles. More than 50% of attached particles are typically lost to this abortive event. The site of uncoating, whether at the cell surface plasma membrane or acidic endocytic vesicle, may be determined by the stability of the virus-receptor complexes. For some serotypes whose receptor complexes are labile at neutral pH (62), binding of the virus to the receptors on the cell surface is sufficient to trigger uncoating. For the serotypes whose receptor complexes are stable at neutral pH (62), some additional stimulus such as low pH is required, so uncoating is delayed until the complexes are transported into the endosomes by normal cellular internalization and trafficking process of receptors and their ligands (47). Clathrin-mediated endocytosis is the major pathway of all cells to internalize receptor-ligand complexes. Both major and minor group RVs use this pathway to enter the endosomes via the interaction of the cytoplasmic domains of their receptors with clathrin (63, 64).

Translation (step 4) is the crucial first step of viral multiplication because synthesis of new viral RNA cannot begin until the necessary viral proteins are made. By using the IRES to appropriate ribosomes and other components of the protein synthesizing machinery from the host cell, the incoming viral RNA strand directs synthesis of a polyprotein that is progressively cleaved into the final products already described. RV translation requires multiple cellular translation proteins, including most of the canonical translation initiation factors, with the exception of the cap-binding protein eIF4E (eukaryotic translation initiation factor 4E) and three additional cellular RNA-binding proteins, PTB (polypyrimidine-tract binding protein), PCBP2 (poly(rC)-binding protein 2), and unr (upstream of N-ras). PTB, PCBP2, and unr interact with IRES to help it to bind directly to the cellular translation machinery.

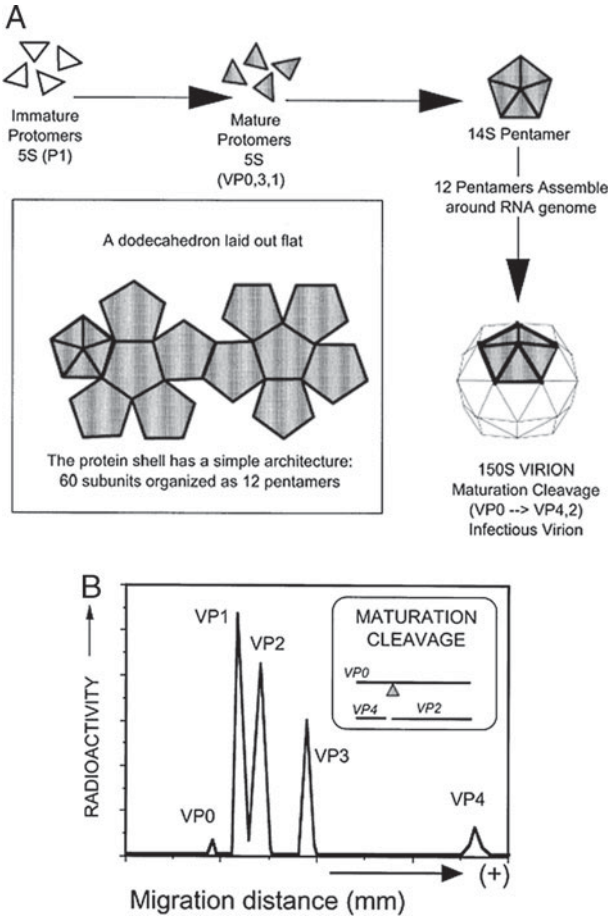

FIGURE 4 (A) Diagram representing virion architecture and assembly. (B) The mature virion contains four major proteins (VP1 [1D], 2 [1B], 3 [1C], and 4 [1A],) plus traces of VP0, representing residual precursor following the maturation cleavage required for acquisition of infectivity.

The initial step in synthesis of new viral RNA is copying of the incoming genomic RNA to form complementary minus-strand RNA (step 5), which then serves as the template for synthesis of new positive-strands (step 6). Synthesis of positive-stranded RNA, which occurs in viral-induced membranous vesicles derived from endoplasmic reticulum, is initiated rapidly to generate multistranded replicative intermediates consisting of one minus-stranded template and many nascent positive-stranded copies. The initiation of minus-strand synthesis is apparently down-regulated by the presence of translating ribosomes on the positive-strands (65). Generally, an infected cell has 30 to 70 times more positive-strands than minus-strands (66).

A molecule of VPg is covalently linked to all RNAs involved in transcription. The role of VPg in the initiation of minus-strand RNA transcription has been elucidated for poliovirus. Following uridylylation by the 3D polymerase, VPg-pUpU primes the transcription of poly(A) RNA to produce VPg-linked poly(U) RNA. The template for the uridylylation reaction is a small RNA hairpin, called the cis-acting replication element (cre), located in the coding region of protein 2C gene of poliovirus (67). Cre hairpin structure is also present in the RNA genomes of RVs. Unlike polioviruses, cre hairpin structure is located in the coding region of 2A, VP1 and the VP2 genes of RV-A, RV-B, and RV-C viruses, respectively (33, 68, 69).

In the early stages of RNA synthesis, newly synthesized positive-stranded RNA molecules are recycled to form additional replication centers (step 7) until, with an ever-expanding pool of positive-stranded RNA, an increasing fraction of the positive-stranded RNA in the replication complex is packaged into provirion. The RNA replication machinery interacts directly with the capsid proteins so that only newly synthesized positive-stranded viral RNAs are packaged (70).

Virion assembly (steps 8 to 10) is controlled by a number of events. Before assembly can begin, coat precursor protein P1 must be cleaved to form mature protomers composed of three tightly aggregated proteins (VP0, 3, and 1). Early in the infection cycle this cleavage is likely very slow because of the low concentrations of P1 and its protease. Later, with increasing protease activity, the rising concentration of mature (5S) protomers triggers assembly into pentamers that then package the newly synthesized VPg-(+)RNA to form provirions. Formation of infectious 150S to 160S particles results from the final maturation cleavage (61). Completed virus particles are ultimately released by infection-mediated disintegration of the host cell (step 11).

To better exploit cellular factors for its own reproduction, RV uses its proteases 2A and 3C to inhibit host transcription and translation. The 2A protease reduces the efficiency of host-cell translation of capped mRNA via the cleavage of

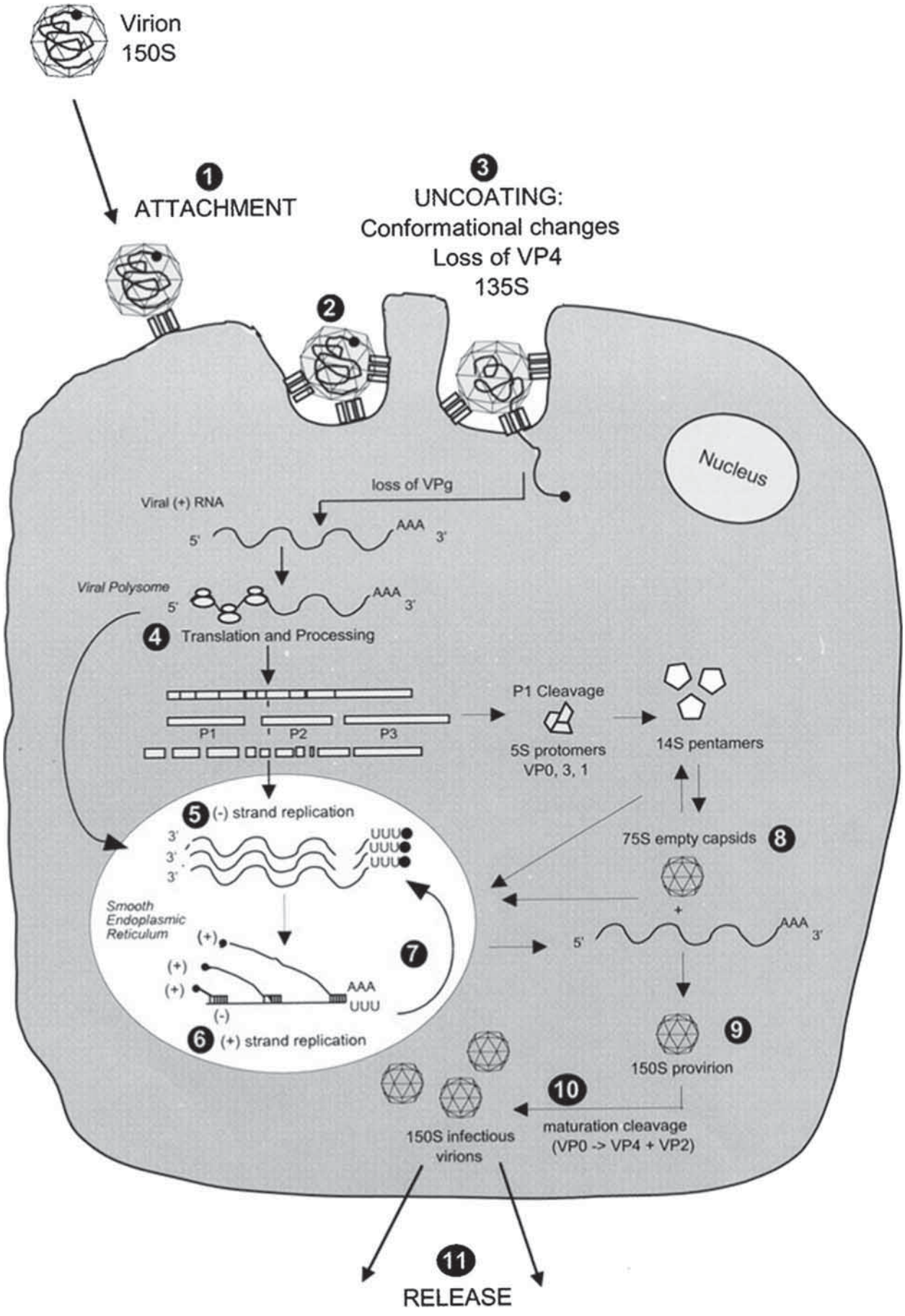

FIGURE 5 Overview of the RV infection cycle.

the eIF4G subunit of the cap-binding protein complex. The cleavage separates the N-terminal eIF4E (cap-binding protein) binding domain from the C-terminal fragment (p100) that has the binding sites for IRES and the translation machinery and thus reduces the ability of capped mRNA to compete with viral mRNA for translation (71). The 3C protease and its precursor 3CD inhibit transcription of host RNA polymerases I, II, and III via the degradation of transcriptional accessory proteins in the nucleus. Because 3C lacks a nuclear localization signal (NLS), it enters the nucleus in the form of its precursor, 3CD, that has an NLS in 3D.

Host Range

Human RVs have a high degree of species specificity. The natural host range of RV-A and RV-B viruses is restricted to humans and closely related apes. Early efforts to seek an animal model found that the chimpanzee and gibbon are the only two nonhuman hosts that can be productively infected with RV-A and RV-B viruses (72, 73). Chimpanzees and gibbons that were exposed intranasally to RV-A or RV-B shed large amounts of virus for several days, produced specific neutralizing antibody, and were protected when rechallenged with the same virus, although they did not have clinically detectable symptoms (72, 73). The nonhuman host range of RV-C has not been reported.

Mouse Model

Genomic RNA of a number of RV serotypes, such as 1A, 1B, and 16, can replicate in mouse cells to yield some infectious progeny virus (72–77). Encouraged by these findings, investigators have developed mouse models for studying RV infection: intranasal inoculation of transgenic mice expressing human ICAM-1 with major group RV16 and of nontransgenic mice with minor group RV1A or RV1B. Some studies have reported the observation of inflammatory changes, similar to those induced by RV infection of human airways, including neutrophilic and lymphocytic inflammation, mucin secretion, and cytokine production when a large virus inoculum (typically 5×10^6 $TCID_{50}$ units per mouse) was used. However, none of these studies has demonstrated convincing evidence of viral replication in mice (78–82).

Growth in Cell Culture

Primary WI-38 (ATCC CCL-75), MRC-5 (ATCC CCL-171), and continuous H1 HeLa cells (ATCC CRL-1958) are the most commonly used cells for studying live RV-A and RV-B viruses (Fig. 6). WI-38 and MRC-5 are strains of human embryonic lung fibroblast diploid cells and are sensitive to the prototype strains of all 100 RV-A and -B serotypes, as well as several other human viruses. They are useful for isolating RV-A and -B viruses from clinical samples, titrating their infectivity and producing virus inocula for human studies (83). They have a finite passage life of about 50 cell divisions, however, and require a solid substrate for growth. Recently, a master cell bank from passage one of a well-characterized strain of human diploid embryonic lung cells (WisL cells) was created under good manufacturing practice (GMP) conditions (Waisman Clinical Biomanufacturing Facility, University of Wisconsin-Madison) for producing RV inocula for human studies. These WisL cells have passed the tests required by FDA for cell lines used to produce biologicals for human use.

H1-HeLa is an RV-sensitive HeLa strain originally developed as a monolayer culture in the lab of Vincent Hamparian in the early 1960s. It has been adapted for

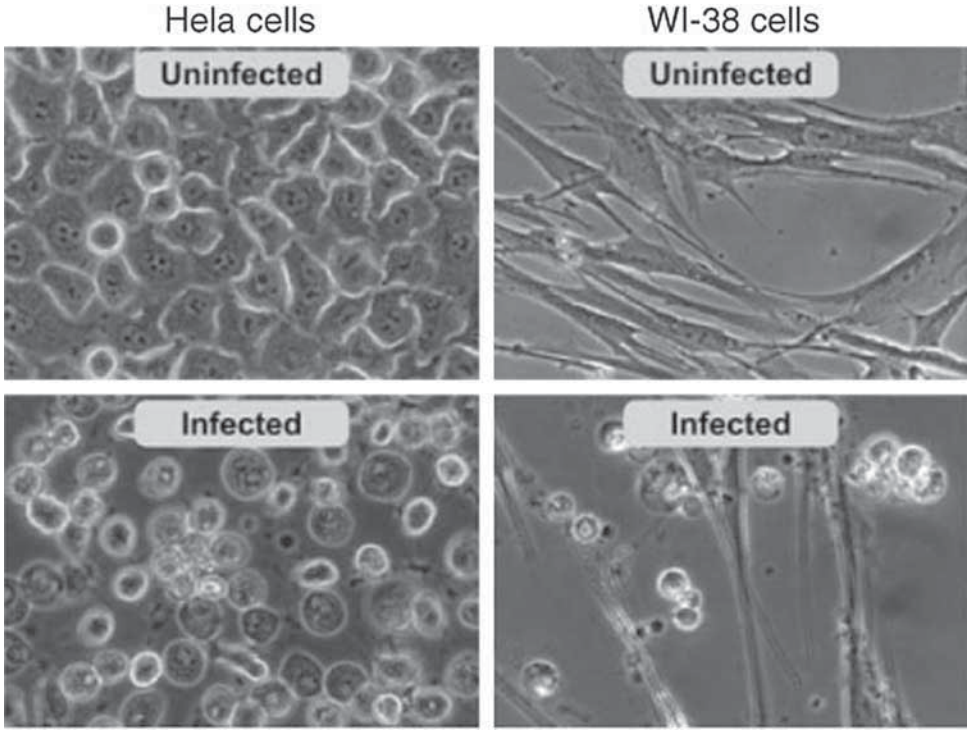

FIGURE 6 Cytopathic effect (CPE) in H1-Hela and WI-38 cells caused by RV infection. Cells were exposed to 100 PFU of human RV type 16 (RV16) per cell and incubated at 35°C. Infected HeLa cells became rounded and detached from growth surface by 12 hours but WI-38 cells required 48 hours to develop CPE.

growth in suspension culture (46). H1-HeLa cells support vigorous multiplication of HeLa-adapted serotypes and are also the standard cell for plaque assays of RV-A and -B viruses (83). However, this cell line is derived from a human cervical carcinoma and is therefore unsuitable for preparing virus inocula for studies in human volunteers. In cultured H1 HeLa cells, the time required for a replication cycle of RV16, from infection to completion of virus assembly, is about 6 to 8 hours at 35°C with a yield of about 300 plaque-forming units per cell (46, 54).

In vitro differentiated human tracheobronchial epithelial cultures have also been exploited as a model for studying RV infection of the native airway (84). These cultures were developed by differentiating primary airway epithelial cells, isolated from human tracheobronchial tissues, in air-liquid interface cultures to form a polarized mucociliary epithelium with structural characteristics similar to native airway tissues (85).

RV-C grows only in human sinus organ culture, differentiated airway epithelial cells in air-liquid interface culture and HeLa cells transfected with its putative RV-C receptors (38, 39, 41).

Inactivation by Physical and Chemical Agents

When subjected to any of a variety of treatments including gentle heating, ultraviolet light, high pH, mercurials, phenol, or desiccation, rhinovirions lose their native (N) antigenicity and acquire a new set of surface determinants called the C (coreless) or H (heated) antigen. RVs are characteristically sensitive to acidic pH less than about 5 or to alkaline pH exceeding 9. Increasing ionic strength generally increases thermostability. Capsid-binding inhibitors markedly stabilize RVs against heat inactivation (86). Their thermostabilizing effect has been attributed to either increasing rigidity of the coat proteins or to increasing compressibility of the capsid. Susceptibility of RV-C to thermal, osmotic, and pH stress resembles that of RV-A (87).

Sodium dodecyl sulfate, particularly in the presence of organic acids such as malate and citrate, inactivates RVs rapidly even at room temperature. Infectivity of aqueous suspensions of RV survives extraction with diethyl ether, a treatment commonly used for the selective killing of bacterial and fungal contaminants and as a preliminary test to distinguish lipid-enveloped viruses from nonenveloped ones. Infectivity is less refractory to extraction with more polar organic solvents such as chloroform, possibly because of the removal of the hydrophobic "pocket factor," partial solvent denaturation of the protein shell, or oxidation due to contamination of the solvent by phosgene. Chemicals that alter the nucleic acid include nitrous acid, which deaminates purine and pyrimidine bases, and alkaline reagents including ammonia, which cleaves RNA within the virus particle. Halogens (chlorine, bromine, iodine), hydrogen peroxide, and ozone are also commonly used disinfectants, and one exploratory study used an iodine hand treatment in efforts to inhibit transmission (88).

EPIDEMIOLOGY

Geographic Distribution

RVs are distributed worldwide, and antibody to RV is detected in serum specimens collected from many different parts of the world. Of particular interest has been the detection of RV antibody in serum specimens collected from members of an isolated Amazon Indian tribe shortly after their initial contact with civilization (89). Infection in this group was presumably acquired through intermittent contact with semicivilized tribes in the same region.

Incidence and Prevalence of Infection

Recent studies using reverse transcriptase PCR (RT-PCR) for detection indicate that RV infections are ubiquitous, especially in young children. In 15 healthy children followed for three seasons with weekly nasal swabs, the overall rate of infection was 0.5 infections per month (90). Highest rates were in the fall (0.63 infections per month), and lower rates were present during summer and winter (0.43 and 0.44 infections per month respectively). In a family surveillance study involving 108 persons from 26 households, analysis of weekly samples collected over a year demonstrated that 93% of participants had at least one RV infection detected (91). Asymptomatic RV infections were common but varied by age. Infections in children younger than 5 years were less likely to be asymptomatic compare to all other age groups (33% vs. 49%) (91). Detection of RV in asymptomatic individuals can be due to a recent infection after symptoms have resolved, individuals who are prodromal, and true asymptomatic infection (92). With the exception of immunosuppressed individuals, persistent infection with a single RV type does not occur (92–95). The relationship between infection and illness severity appears to vary by season; infections of infants in the winter are about 10-fold more likely to cause moderate-to-severe colds compared to infections during the summer months (96).

In the absence of protective antibody, the risk of infection with a new RV type is primarily a function of exposure to the virus. The prevalence of RV-specific antibody in serum indicates that infection with the various serotypes begins in early childhood and continues throughout life. The peak prevalence of RV antibody is found in young adults, probably reflecting exposure to young children in the home, and the subsequent decline in prevalence is probably related to less frequent exposures in older adults. Type-specific neutralizing antibody provides serotype-specific protection, but this is associated with little reduction in overall infection risk due to the large number of RV types.

Seasonality

Frost and Gover (97) suggested that the respiratory disease season is composed of "successions of epidemics" caused by different infectious agents, based on epidemiological observations made between 1923 and 1925. This ingenious observation was confirmed with the subsequent detection of the several families of respiratory viruses. In temperate areas of the northern hemisphere, a distinct peak of colds is observed in September (97, 98). This early-fall peak of colds is strongly associated with RV infections, a finding confirmed by studies using culture or RT-PCR. In young adults the RV infection rate reaches its highest annual point (3.5 illnesses/1,000 persons/day in Charlottesville, VA during this period (99). Thereafter, RV prevalence declines, usually remaining low throughout the late fall, winter, and early spring (90). A second period of increased RV activity occurs in late spring, April and May. Although the overall incidence of colds is low during the summer months, RV infections account for up to 50% of the illnesses during this season (90, 99). In the temperate areas of the southern hemisphere, the seasonal incidence of infection mirrors that in the northern hemisphere. In tropical climates, RV activity is detected throughout the year, with a peak incidence in the autumn months (100).

The cause of the seasonality of colds has been the subject of much speculation but remains largely unexplained. However, at least two factors influence this phenomenon: the effect of relative humidity on virus survival and the herding of children during the school term (101, 102). RV and other nonenveloped viruses retain infectivity best in conditions of high relative humidity, in contrast to enveloped viruses like influenza virus. In 1960 Hemmes et al. (103) suggested that the relative humidity indoors is an important factor controlling the seasonal fluctuations of the different virus families.

Observations suggest that weather has both biological and behavioral consequences that influence the incidence of RV infection. In a 15-year longitudinal study in Charlottesville, VA, the occurrence of colds with culture-detected RV was greatest from April to October, which is the period when indoor relative humidity tends to remain above 45% (101). School openings also correlated with the fluctuating incidence of colds. In 8 of 14 years, the interval between the date of school opening and the date on which cold rates reached the defined peak was 11 to 14 days. School openings also precede fall exacerbations of asthma, which are usually associated with RV infections (104).

Transmission

RV infection is readily initiated by inoculation of virus onto the nasal mucosa or conjunctiva where transport by the lacrimal duct leads to deposition in the posterior nasopharynx (105). In those lacking serotype-specific neutralizing antibody, the 50% human infectious dose (HID_{50}) has ranged from 0.1 to 6 $TCID_{50}$ after intranasal inoculation. In contrast, experimental inoculation of RV into the mouth or exposure to infected volunteers by prolonged kissing is an inefficient method of initiating infection (106). A similar difference in susceptibility is observed between the upper and lower respiratory tracts. The HID_{50} for antibody-free volunteers given RV type 15 by nasal drops corresponded to 0.032 $TCID_{50}$, compared to 0.68 $TCID_{50}$ when the virus was given by small-particle aerosol (107). This 20-fold disparity in infectious dose between nose drops and inhaled particles suggests that the lower respiratory tract is less susceptible to RV infection than is the nasopharynx. In addition, interferon applied intranasally by drops or coarse spray prevents natural RV infection and illness (108). Since application of interferon by this method would not be expected to prevent infection in the lower airway, this also points to the upper airway as the usual portal of entry for RV.

Delivery of RV to the nasal mucosa can occur either by droplets or by direct contact. Sneezes and coughs generate both large- and small-particle aerosols, but the amount of RV in respiratory secretions produced by coughs and sneezes is usually small. Although the RV genome may be present in small-particle aerosols, such aerosols do not appear to be an important mechanism of spread. Large-particle aerosols produced by coughs and sneezes and deposited onto the nasal or conjunctival mucosa contribute to transmission (109). RV is recovered from the fingers of approximately 65% of experimentally infected volunteers after finger-to-nose contact, and the virus survives for several hours on skin (110, 111). Contact with the contaminated fingers reliably transfers virus to the skin of a recipient individual. Once the fingertips of the recipient are contaminated, infection is readily induced by self-inoculation of the nasal mucosa by rubbing the nose or the eyes.

The role of fomites in the transmission of virus is less clear. The virus contaminating the hands is readily transferred to objects in the environment. When fingertips have been experimentally contaminated with nasal mucus containing known amounts of RV, 13% of the starting virus titer was transferred to inanimate objects after brief contact, although transfer was less efficient if the virus inoculum was allowed to dry (110, 111). Virus on nonporous environmental surfaces may survive for up to 3 to 4 days and can be transmitted to the skin by contact. Although these various steps in fomite transmission have been demonstrated, there is a substantial loss of infectious virus at each step. An attempt to document transmission of infection from fomites under experimental conditions that would favor transmission was unsuccessful, suggesting that this mechanism may not be efficient for the spread of RV infections (112).

Studies in the natural setting have confirmed many of these steps in virus transmission. Virus is recovered from the skin of the hands of approximately 40% of individuals with natural RV colds and from 6% to 15% of objects in their environment (110, 111). Individuals in the natural setting routinely make finger-to-eye or finger-to-nose contact in a manner that would transfer virus to the nasal mucosa. However, the mechanism of transmission of virus under natural conditions can be determined only by blocking transmission using an intervention that is specific for a particular route. In one trial using a 2% aqueous iodine as a virucidal treatment for the hands (110), regular applications to the fingers by mothers who had been exposed to a child with a fresh cold in the home reduced colds by 67% compared to placebo. None of the 11 mothers using iodine became infected with the same RV recovered from the index case, compared to 31% of mothers using placebo. Another study of the hand contact route of cold transmission in asthmatic children (113) found that a group of children trained to avoid finger-to-nose contact had significantly less self-inoculation behavior, fewer viral respiratory infections, and fewer attacks of asthma. Thus, direct evidence is available that supports the hypothesis that RV colds are transmitted by accidental self-inoculation of the nose or conjunctiva following inadvertent contamination of the fingers with virus.

The duration of infectiousness for RV colds parallels the period of maximum virus shedding in nasal secretions. RV concentrations in nasal secretions are highest during the second and third days after experimental infection. Deliberate infection of one member of a dually susceptible (i.e., antibody-free) married couple (114) determined that the features of the donors that were associated with transmission included a concentration of >1,000 $TCID_{50}$ per ml nasal washings, virus on the hands and anterior nares, and symptoms of at least a moderately severe cold. In most instances, these conditions occurred only on the second and third days after virus inoculation. In households, secondary cases are seen 1 to 3 days after exposure to an index case. In longitudinal studies within families, transmission from children to other children and adults is much more common than transmission from adults to children or to other adults (115, 116).

PATHOGENESIS IN HUMANS

Incubation Period

In experimentally infected volunteers, RV is first recovered in nasal washes a median of 10 hours (range 8 to 18 hours) after inoculation. Respiratory symptoms occur surprisingly early, with the throat becoming sore or scratchy between 10 and 12 hours after virus inoculation as newly produced virus

appears (117). Nasal symptoms follow soon afterward, and experimental inoculation studies demonstrate that lower respiratory symptoms, when present, may begin 1 to 3 days following the onset of cold symptoms (118). The incubation period ranges from 1 to 4 days following inoculation and is similar following natural transmission (114).

Patterns of Virus Replication

In studies of natural or experimentally induced colds, RV protein or genome is detected in the epithelial layer (39, 119, 120) and in some subepithelial macrophages (121). Replication does not occur in macrophages (122), although these cells likely mediate immune surveillance by binding virus and transporting it to regional lymph nodes. RV can also bind to monocytes, eosinophils, and fibroblasts (123). RV can replicate in airway fibroblasts in tissue culture (122–124) but whether these cells are infected *in vivo* is speculative. Infection occurs in a patchy distribution, with small foci of infected cells (119), and greatest viral shedding in the nasopharyngeal and adenoidal regions (125). In the adenoids, RV may replicate in nonciliated cells that express high levels of ICAM-1 and resemble intestinal M cells (126). In cultured epithelial cells, ciliated cells are preferentially infected by major group RV (127). The duration of RV shedding is generally limited to 7 to 10 days, but prolonged shedding of the same RV type occurs in a subset (<10%) of infections in young children (93, 128) and in neonates (129).

Because early studies indicated that RV replicates best at 33–35°C, it was assumed that RV infection was limited to the upper airways. Contrary to these initial assumptions, direct measurements in the lower airways have shown that large and medium size airways maintain the ideal temperature for RV replication (130), and many RV types replicate efficiently at 37°C (87, 131). Accordingly, RV replicates readily in cultured lower airway epithelial cells (132, 133). Furthermore, RV can be detected in the lower airway following experimental inoculation of the upper airway (120, 134) and in lower airway secretions of children with natural colds in whom samples are obtained through tracheostomies, which eliminates the potential confounding effect of sample contamination with upper airway secretions (135).

In addition to infecting the nasopharynx and adenoids, conjunctiva, and lower airways, RV can also be detected in specimens obtained from the middle ear and sinuses (136, 137), presumably because the virus has spread from the nasal epithelium to contiguous areas.

Factors in Disease Expression

An impressive feature of RV pathogenesis is that the nonspecific host defense mechanisms of the nose are unable to prevent infection in the nonimmune individual and may, in fact, contribute to the pathogenesis of RV-associated illness. Following intranasal RV challenge, infection rates of more than 90% are routinely achieved in nonimmune volunteers, irrespective of the season of the year or of the allergic status, smoking history, physical condition, or stress level of the subject (138–140). Following infection, symptomatic individuals shed higher titers of virus in nasal secretions than do asymptomatic individuals.

Infection with RV produces no detectable histopathological change in the respiratory epithelium. Nucleic acid hybridization studies demonstrate small foci of infection interspersed among large areas of uninfected cells (141), and examination of nasal secretions with labeled RV antibody demonstrates occasional antigen-positive epithelial cells that have been shed from the nasal mucosa (142). These studies suggest that RV infection involves sparse and scattered areas of the nasal epithelium and that infected cells are shed from the epithelium, with rapid repair of the involved areas.

Despite the absence of overt histopathological damage to the nasal mucosa, inflammatory responses occur in the nasal epithelium and increased numbers of nasal epithelial cells are shed in secretions. Polymorphonuclear leukocytes (PMN) infiltrate the nasal epithelium early after infection, and PMN concentrations increase in the nasal secretions and peripheral blood but only in symptomatic individuals. The correlation between the lymphocytic response to RV infection and symptomatic illness is less clearly characterized. Modest increases in T-lymphocyte concentrations have been reported both for the nasal mucosa and for nasal secretions during RV infection; few B lymphocytes are noted in the nasal mucosa.

The evidence summarized above suggests that nonspecific host inflammatory responses may play a role in symptom expression (Fig. 7). A number of inflammatory mediators are increased in nasal secretions and/or the serum during RV colds, including bradykinin, interleukin (IL)-1b, IL-6, IL-8, interferon-inducible protein 10, and tumor necrosis factor alpha. The concentrations of IL-6 and IL-8 in nasal secretions have some correlation with symptom severity, and intranasal instillation of IL-8 in healthy volunteers produces symptoms that are similar to the common cold. Inhalation of bradykinin by healthy volunteers produces nasal obstruction, rhinorrhea, and sore throat. The detection of these mediators in association with illness, however, does not provide definitive evidence of a role in RV-associated illness. The role of kinins, in particular, is unclear in light of a study demonstrating that inhibition of the kinins with corticosteroids had no effect on cold symptoms (143).

Neurogenic mechanisms, particularly the parasympathetic nervous system, also appear to play a role in the expression of illness during RV infections (Fig. 7). The parasympathetic nervous system controls the secretory activity of nasal seromucous glands. These glands, in association with plasma transudation, provide most of the nasal fluid produced during an RV cold. Drugs with anticholinergic activity, such as atropine methonitrate and ipratropium bromide, given intranasally and first-generation antihistamines given orally reduce mean nasal fluid volumes by approximately 30% in volunteers with experimental RV colds. The mechanisms by which these mediators and neurogenic reflexes are stimulated by RV infection remain unknown. In addition to the obvious interaction between RV and the known cellular receptors, other nonspecific interactions may play a role in pathogenesis. Toll-like receptor 3, which recognizes double-stranded RNA, appears to mediate some cellular responses to RV (144). RV replication complexes occur in ceramide-enriched cell membrane platforms that may play a role in stimulating relevant signaling pathways (145). A number of secondary signaling pathways have been implicated in the elaboration of inflammatory mediators *in vitro*, but the relevance of these studies to the *in vivo* elaboration of mediators remains to be determined.

Other host factors include possible roles of the host psychological state, sleep patterns, and genetics in promoting infection or illness. Shorter duration of sleep has been associated with an increased risk for developing cold symptoms after experimental RV inoculation (146). Stress is not a factor in the acquisition of infection, but chronic stress in particular is associated with the development of more severe symptoms (147). Genetic factors related to RV illness

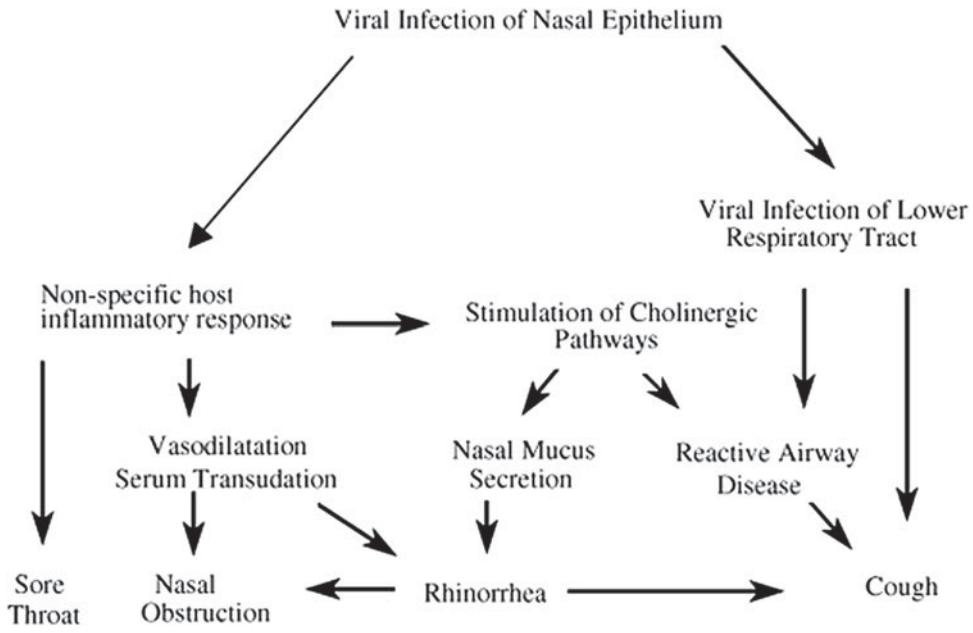

FIGURE 7 Proposed pathogenesis of symptoms associated with RV infection.

severity include polymorphisms in regions related to antiviral responses (e.g., *IFNB1*, *JAK2*, *IL28A*), immunoregulation (e.g., *IL6*), and the vitamin D receptor (148, 149).

Immune Responses

Individuals who develop symptomatic RV illness have a prompt inflammatory response, with elaboration of inflammatory mediators and PMN infiltration. Whether these inflammatory responses help to control the infection is uncertain. Peak virus shedding occurs at approximately the same time as the peak of symptoms, and virus concentrations in secretions fall coincidently with the resolution of symptoms. Low levels of virus shedding can continue for 2 to 3 weeks, however, and the termination of viral shedding and protection from subsequent infection are most closely correlated with the appearance of neutralizing antibody. Serum neutralizing antibody titers rise in 40% to 80% of persons following natural or experimental RV infection, depending on the serotype involved. The reason for the differences in antigenicity of the various RV serotypes is unknown. Studies using serial specimens collected from the same individuals have shown that serum neutralizing antibody can persist for years. Serum neutralizing antibody levels of ≥8 are associated with good protection following natural exposure in the home, and levels of ≥16 are generally associated with solid immunity.

Although it appears that neutralizing antibody is important in elimination of RV shedding from nasal secretions, recovery from illness and the initial reduction in the viral load in nasal secretions occur before specific antibody appears. Type I interferons likely contribute but are detected in the nasal secretions during only one third of infections. Preinfection gamma-interferon concentrations are inversely correlated with both severity of illness and duration of virus shedding during experimental colds (150). Type III interferons are more readily detected in nasal secretions and have anti-RV effects *in vitro* (151), but their role in clinical illness is not well understood.

Several mechanisms for antibody-mediated neutralization of RVes have been proposed, including aggregation, virion stabilization, induction of conformational changes, and abrogation of cellular attachment (58, 59). Taken together, numerous biochemical and structural studies suggest that neutralization of RV *in vitro* results from steric blockage of the interaction between the virus and its receptors due to the binding of the antibody molecules on the virion surface (59).

The relative importance of serum versus nasal neutralizing antibody for protection has been difficult to determine, because serum antibody is found in close association with nasal antibody. The ratio of nasal to serum antibody following recent infection is in the range of 1:2. Over time the ratio falls to the range of 1:16, apparently because nasal antibody concentrations decline more rapidly than in the serum.

The dynamics and the role of neutralizing antibody during acute illness vary depending on the antibody status of the host. During the acute phase of RV colds, there is considerable transudation of serum proteins into nasal secretions (152). In seronegative persons, RV neutralizing antibody appears in serum and nasal secretions approximately 2 weeks after onset of infection. The neutralizing activity is present in the immunoglobulin A (IgA) and IgG antibody classes, as well as in IgM in early infection. Neutralizing titers rapidly increase during the third and fourth weeks, at which time viral shedding in nasal secretions is no longer detectable (125).

Investigations into cellular mechanisms of innate immunity against RVs indicate that epithelial responses can be initiated by engagement of cell surface receptors to the virus (153, 154), detection of intracellular viral RNA (e.g., PKR, TLR3, and RIG-I) (144, 155, 156), and activation of proinflammatory signaling cascades (e.g., NFκB, p38 MAP kinase) (153, 157). The net result of these processes is increased transcription and production of intracellular antiviral effectors, as well as a host of secreted cytokines, chemokines, interferons, and mediators (158, 159).

Mechanisms of RV-Induced Exacerbations of Asthma

Several factors contribute to the pathogenesis of virus-induced exacerbations of asthma. More severe colds generally provoke exacerbations of asthma (118). Accordingly, RV-A and RV-C cause greater severity of illness compared to RV-B (96, 160), and are more likely to be associated with exacerbations of asthma (20, 161). As in COPD, RV infections in individuals with asthma are often followed by overgrowth of bacterial pathogens, and the combination of RV infection and detection of bacterial pathogens is associated with an increased risk for exacerbation of asthma (162). Whether secondary effects on airway bacteria contribute to the pathogenesis of airway obstruction and clinical symptoms remains to be determined.

Host factors are also important, including allergic sensitization (163), eosinophilic inflammation (138, 164), type 2 inflammatory responses (e.g., IL-5, IL-13, IL-33) (118, 165, 166), and epithelial interferon production (167). The combination of allergic sensitization and allergic exposure increases the risk for exacerbations (168). Interestingly, allergies that are associated with fall exacerbations, most often caused by RVs, are perennial allergens such as dust mite and cockroach instead of fall seasonal allergies (169).

The strong relationship between respiratory allergies and exacerbations of asthma has led to a number of studies into potential mechanisms of synergy between these two factors. There is evidence that respiratory allergies may inhibit interferon responses of blood dendritic cells and monocytes (170, 171). In addition, allergy can interfere with antiviral responses by affecting transcriptions factors (e.g., suppressor of cytokine secretion-1, FoxA3) that influence cell differentiation and the antiviral response (172, 173). Recently, in children susceptible to exacerbation, treatment with omalizumab, which blocks attachment of IgE to high-affinity IgE receptors, demonstrated that focused inhibition of IgE led to improved blood cell virus-induced interferon responses and reductions in total and virus-induced exacerbations of asthma (174, 175).

CLINICAL MANIFESTATIONS

The major clinical syndrome associated with RV infection is rhinosinusitis, which is traditionally characterized as "the common cold." RV colds frequently begin as a sore or scratchy throat that is followed closely by development of nasal obstruction and rhinorrhea. Over the course of the illness, the signs and symptoms of RV colds typically include various combinations of sneezing, rhinorrhea, nasal obstruction, facial pressure, sore or scratchy throat, hoarseness, cough, headache, malaise, and feeling chilly or feverish. Cough occurs in approximately 30% of colds; it frequently appears after the onset of nasal symptoms and often persists longer. The clinical features of RV colds are similar in adults and older children. Infants and young children may at times display only mucus discharge from the nose. In addition, infants more likely to have fever, which is uncommon in infected children and adults, and less likely to have asymptomatic infection (116, 176).

The median length of natural RV colds is 7 days, with approximately one fourth lasting 2 weeks. However, their duration is quite variable and has ranged from 1 to 33 days in prospective epidemiological studies (177). Resolution of the most severe symptoms occurs quite rapidly in most cases, and lingering minor symptoms generally account for the prolonged duration of illness reported by some individuals.

Acute Sinusitis and Otologic Changes

RV has been recovered from sinus aspirates of patients with acute community-acquired sinusitis, and RV RNA has also been detected in brushings from the sinus cavities of similar patients. Sinus imaging studies show that sinus involvement is an inherent feature of colds; thus, a RV cold is a viral rhinosinusitis. In young adults with early self-diagnosed common colds, sinus cavity abnormalities have been observed on computed tomography in up to 87% of patients (178). These individuals recover from their illness without intervention.

RV infection also results in abnormalities of the eustachian tube and middle ear (179, 180). Abnormalities in middle ear pressures are seen in 40% to 75% of volunteers with experimental RV colds and 72% to 76% of patients with natural RV colds, sometimes in association with middle ear effusions. RV has been recovered alone and in combination with bacteria in middle-ear fluids from 24% of patients with otitis media (181). It is unclear whether viral invasion of the middle ear is required for the development of the eustachian tube and middle ear abnormalities.

Laboratory Abnormalities

Routine laboratory tests are not useful in the clinical evaluation of patients with suspected RV colds. During experimental RV infection, there is a modest increase in blood neutrophils, and a moderate elevation of the erythrocyte sedimentation rate in some volunteers. A predominance of PMN in the nasal secretions is characteristic of uncomplicated colds and does not aid in the diagnosis of bacterial superinfection. Nasal resistance is increased and nasal mucus transport times are mildly decreased in patients with natural and experimental RV colds. Because sinus and nasal cavity computed tomography scans show abnormalities (178), the latter tests are not appropriate or useful in the clinical management of patients with RV colds.

Complications

Acute Bacterial Sinusitis

The incidence of secondary acute bacterial sinusitis is difficult to ascertain given the changes that occur in the sinuses in uncomplicated RV colds. Various reports have estimated that 0.5% to 8% of viral colds are complicated by bacterial sinusitis. The factors leading to secondary bacterial invasion of the sinus cavity during colds are incompletely understood. Nose blowing propels nasal secretions into the sinuses, and occlusion of the ethmoid infundibulum in many patients with colds may trap nasopharyngeal bacteria in the sinus cavity, thus leading to secondary bacterial infection.

Acute Bacterial Otitis Media

Acute bacterial otitis media complicates an estimated 2% of colds in adults and up to 30% of colds in children. A high incidence of eustachian tube dysfunction during the common cold has been reported, and bacteria presumably reach the middle ear by a mechanism similar to that described above for sinusitis.

Exacerbations of Chronic Obstructive Lung Disease (COPD)

Up to 40% of exacerbations of COPD have been associated with respiratory viral infections, most commonly caused by RV (182, 183). The episodes are characterized by fever,

increased purulence of the sputum, and worsening of ventilation. Transient decreases in pulmonary function have occurred in patients with chronic obstructive pulmonary disease experiencing natural RV colds and following experimental inoculation with RV (184, 185). The pathogenesis of these abnormalities likely involves direct viral invasion of the large airways, which may facilitate overgrowth of proteobacteria including bacterial pathogens such as *H. influenzae* (186).

Exacerbations of Asthma

RV is the principal virus implicated in precipitating asthma attacks in older children and adults and is associated with 60% to 90% of the asthma exacerbations in children (187, 188). Both a fall peak of asthma exacerbation and the fall increase in the incidence of RV infection have been reported in association with the start of the school year (102, 104). RV is particularly important in precipitating episodes of asthma in children over 2 years of age, in whom the infection is often associated with allergen-specific IgE (163), and the timing of the asthma peak may be due to a convergence of RV infection spread among schoolchildren and seasonal allergen exposure (168).

Other Lower Respiratory Syndromes

RV is often detected in upper airway secretions in children with pneumonia (20, 189, 190). RV-associated lower respiratory syndromes are most common in very young children, and RV is the second most common pathogen associated with infant bronchiolitis (191, 192). RV-C and RV-A are more likely associated with lower respiratory symptoms than RV-B (20). Given the difficulty in obtaining lower respiratory specimens, it is uncertain how often RV is a sole pathogen vs. serving as an initiating factor for secondary bacterial infections. In wheezing infants, RV has been detected in lower airway biopsies, and RV detection was associated with reduced lung function in these infants (193). One study reported that 24% of children with pneumonia had RV detected in the upper respiratory tract by RT-PCR (189), although over one half of these patients had evidence of a concurrent bacterial infection. Seasonal trends for invasive pneumococcal disease in young children correlate with RV prevalence, suggesting an interaction (194). In adults, RV is the virus most often detected, either alone or more commonly associated with a bacterial pathogen, in patients requiring ventilation for severe pneumonia (195, 196).

RV infections have also been implicated in exacerbations of chronic lung symptoms in cystic fibrosis (CF) (197, 198). Lower airway antiviral responses may be impaired in CF, as viral load in lower airway secretions in CF exceeds that of samples from patients with asthma, or samples from normal individuals (199).

Infections in Immunocompromised Patients

RV infections can be problematic in adults and children with immune deficiencies or in those who are immunosuppressed following bone marrow or organ transplantation (200, 201). For example, weekly surveillance of 215 patients following bone marrow transplantation demonstrated a cumulative incidence of RV infections of 22% in the first 100 days following transplantation (202). Prolonged viral shedding was detected in 6 of 45 patients, but only 2 developed lower respiratory illness. Furthermore, persistent infection of the lower respiratory tract with the same RV type can occur (203), and viral load correlates with severity of symptoms in immune compromised individuals (204). Adults with X-linked agammaglobulinemia (XLA) have more frequent and prolonged infections and illnesses with RV, even when taking appropriate immunoglobulin replacement (205). *BTK*, which is the defective gene in XLA, contributes to signaling through TLR8, which detects intracellular single-stranded RNA. This may explain the increased susceptibility of patients with XLA to infections with both RVs and enteroviruses (205).

When RVs are detected in lower airway secretions, interpretation of the virology should be tempered by knowledge of the high prevalence of RV in the general population, the potential for contamination of the lower airway specimens by upper respiratory secretions, and the possibility of concurrent infection with a respiratory pathogen, lending support to the concept that the virus can cause prolonged viral shedding and clinically meaningful infections in the context of immune suppression.

CLINICAL DIAGNOSIS

Colds are familiar to everyone, and the illness is usually self-diagnosed before the patient seeks medical attention. The physical findings in the common cold are limited to the upper respiratory tract. Increased nasal secretion is frequently obvious, and a change in the color or consistency of the secretions is common during the course of the illness and is not an indication of sinusitis or bacterial superinfection. Examination of the nasal cavity may reveal swollen, erythematous nasal turbinates; however, this finding is nonspecific and of limited diagnostic usefulness.

Colds are different from episodes of allergic rhinitis; persistent sneezing, thin nasal discharge, watery eyes, and sensation of mucosal itch are more common in the latter. Also, other symptoms of colds such as sore throat, cough, malaise, and headache are less common with allergic or vasomotor rhinitis. When viral culture and nasal smear eosinophilia were used as criteria for diagnosis, colds could be reliably distinguished from allergic rhinitis in adults.

The clinical features of RV infection do not allow reliable differentiation from respiratory infections caused by other viral pathogens. Knowing the seasonal prevalence of the different respiratory viruses helps in suspecting the specific viral etiology of a cold, but a firm diagnosis depends on viral culture or serology, which is usually not practical or necessary for routine patient care.

Distinguishing the rhinosinusitis of an RV cold from a secondary acute bacterial sinusitis is often difficult. Two clinical presentations of acute bacterial sinusitis can be recognized. First, the classical features of acute bacterial sinusitis include fever and facial pain, swelling, or tenderness. There may also be maxillary toothache if the infection is of dental origin. The features of this presentation, while specific, are often not present and thus lack sensitivity. The second and more common presentation of acute bacterial sinusitis is that of an acute respiratory illness which begins as a cold or "flu" but lasts longer than expected. Most natural RV colds have ended by 12 to 14 days, and almost all colds have improved by the second week of illness (177, 206). Therefore, the diagnosis of secondary bacterial sinusitis should be suspected in acute respiratory illnesses that have not improved or are worse after 10 days. Radiographic imaging for diagnosis of bacterial sinusitis is of limited utility (178), and the imaging abnormalities seen with viral and bacterial sinusitis are often indistinguishable.

LABORATORY DIAGNOSIS

Virus Isolation

Human RVs are found in airway secretions, with the highest concentration in nasal fluids. Specimens used for viral culture have been primarily deep nasal swabs or nasal washes. Specimens of respiratory secretions intended for RV culture should be placed in a viral collecting broth that contains proteins, for example BSA, to prevent virus loss due to nonspecific adsorption to plastic or glass surface of the vials.

Historically, cell culture has been the standard method for RV isolation and propagation. Most work has been done in diploid strains of human embryonic lung cells (WI-38 and MRC-5) (83). Diploid fetal tonsil cells and heteroploid cell lines such as HeLa have also been used. Different lots of these cells may vary 100-fold or more in their sensitivity to RV for unknown reasons. RV-C will grow only in sinus organ culture, fully differentiated cultures of primary airway epithelial cells, or in cells engineered to express CDHR3. Therefore, cell cultures should be selected and monitored for sensitivity when used for growing RV.

Most RV types grow best at temperatures of 33–34°C under conditions of motion (e.g., roller drum). Cytopathic effect is readily apparent in sensitive cells (Fig. 6). The 50% human infectious dose (HID_{50}) and the 50% tissue culture infectious dose ($TCID_{50}$) are essentially the same for RV in limited testing (207). One $TCID_{50}$ per 0.5 ml of inoculum was sufficient to cause a productive infection in human without preexisting antibodies (208).

Antigen Detection

Fluorescent antibody and immunoperoxidase methods for detecting RV antigen have been used in experimental studies. However, these techniques are generally serotype specific. Because of the relative difficulty of growing RV in cell culture and the low concentration of RV in respiratory secretions, these methods have not been adapted for clinical use. Immunodetection assay for RV is not commercially available.

Nucleic Acid Detection

RT-PCR-based molecular assays have become the standard tools for the detection, identification, and quantification of RVs in clinical specimens because they are more sensitive and faster and easier to perform than traditional culture-based and serological methods. The 5′ NTR of the rhinoviral genomic RNA has several short stretches of sequence that are highly conserved among all RVs. The PCR primers based on these conserved sequences can be used for sensitive and specific detection of RV-A–B, and -C as a group (209, 96). However, these PCR primers also detect closely related enteroviruses (e.g., EV-D68) which can also cause some respiratory illnesses (209). High throughput multiplex RT-PCR assay kits, such as xTAG RVP (Luminex Corporation) and FilmArray RP (BioFire Diagnostics), are commercially available for the detection of RV/enterovirus and other common respiratory viruses in clinical specimens (91, 210, 211). Molecular typing assays have also been developed for rapid identification of individual RV-A, -B serotypes, or RV-C types in clinical samples (25, 96, 212, 213). These assays use very sensitive and specific two-step PCR method to amplify a 390-bp variable region in the 5′ NTR or a 640-bp region of the capsid protein genes VP4-VP2 of the rhinoviral genome for direct sequence determination. The type is then determined by phylogenetic comparisons of the resulting sequences to corresponding reference sequences of the 150 known RV types (213). The 5′NTR assay is very sensitive. It detects as low as 10 copies of RV cDNA per reaction. The VP4-2 assay is about 80% as sensitive as the 5′NTR assay, and is useful for confirming the typing results of the 5′NTR assay (96). In addition, real-time quantitative (q) PCR methods also been established to measure viral loads of all known RV-A and—B serotypes and some RV-C types (213).

In situ hybridization using RV-specific probes has been used to locate the anatomic sites and cell types which support viral replication in the airways of infected subjects (119, 124), making it a useful technique to study pathogenesis.

Serologic Assays

Viral neutralization assay is the standard serologic method for RV-A and -B viruses (207), and these techniques are under development for RV-C. Complement fixation and hemagglutination tests have been developed but have not proven useful. The neutralization assay is used to identify specific viral serotypes and to measure antibody in human serum and nasal secretions. Viral shedding is a more sensitive indicator of infection than serological response with experimental RV infections (207). However, in some studies of natural infection, either procedure alone identified two thirds of the total diagnosed infections, and in family studies, 20%-40% of infections were detected only by serological assays.

Hyperimmune RV antisera have been produced in several animal species (207). Antiserum preparations from goats and calves have contained cytotoxic substances not found in rabbit and guinea pig preparations. Identification of RV serotypes in epidemiologic studies has been accomplished using antiserum pooled by a combinatorial method that has been adapted to a microtitration system. To measure RV neutralizing antibody in human serum and nasal washes, a small inoculum (3.2–16 $TCID_{50}$) of virus should be used in a neutralization assay to provide sufficient sensitivity to detect the relatively low concentration of antibody in these specimens (215). ATCC has a collection of antisera against 89 RV-A and -B serotypes.

A major problem with using the neutralization assay for diagnosis is the existence of 100 RV-A and RV-B serotypes, as well as difficulties with culturing RV-C types. Thus, serological diagnosis is practical only when the serotype of the infecting virus is known or suspected, such as in experimental virus challenge studies or in family studies where a RV has been recovered from a family member.

PREVENTION

The only source of RV is the human airway and possibly sites in the environment contaminated with virus-containing nasal secretions. Thus, it may be possible to lower the risk of infection by avoiding exposure through modifying personal behavior (handwashing, avoiding contacts when infected) and by selected environmental measures. Eradication of virus contaminating the hands by use of virucidal agents is an attractive approach to prevention of RV infection; however, trials with virucidal agents have not led to commercial products (216, 217). Commonly used hand sanitizers containing 62% ethanol are effective for removing RV from the hands (218).

Hope for the development of a common-cold vaccine was considerably reduced when the large number of common-cold viruses was discovered in the 1950s and 1960s. The application of new technology to identify shared B-cell

epitopes among RV types has led to renewed interest in an RV vaccine (219–222). To date, immunization of mice with cross-reactive epitopes has led to a limited breadth of cross-protection (220). There are also some shared T-cell epitopes among RV types (223), and some evidence that T-cell proliferative responses confer some protection against illness (224).

Chemoprophylaxis of RV infections is an important goal, especially for young children, the elderly, and patients with chronic respiratory disease. Progress in the development of antiviral medications that could be suitable for prophylactic use is described in the next section.

TREATMENT

Symptomatic Therapies

The current treatment of RV-associated illness relies on remedies directed at specific symptoms. For common colds the efficacy of treatments for nasal obstruction, rhinorrhea, and the pain symptoms (i.e., sore throat and headache) has been demonstrated in studies done with adults (225). Attempts to demonstrate beneficial effects of these agents in children have failed, although it is not clear whether this failure is due to a lack of effect in children or simply due to the difficulty in assessing subjective symptoms in this population.

Adrenergic agents given either by topical intranasal administration or orally have demonstrable effects on nasal obstruction. Topical administration of phenylephrine, oxymetazoline, or xylometazoline produces a prompt and marked reduction in nasal obstruction, with a gradual return to the baseline over a period of several hours. The use of these agents may be associated with nasal irritation, and prolonged use may be associated with rebound nasal obstruction. Although there has been no systematic comparison of the effect of topical and oral adrenergic agents on the nasal obstruction associated with the common cold, the oral agents produce about a 20% reduction in obstruction severity, compared to about 80% with the topical agents. The oral agents may also be associated with systemic side effects, including irritability and insomnia, and are contraindicated in children.

Rhinorrhea can also be treated topically or systemically. The first-generation antihistamines are the most commonly used treatment for runny nose and reduce rhinorrhea severity by approximately 25%. The effect on rhinorrhea appears to be related to the anticholinergic effects of the first-generation antihistamines, since the second-generation (nonsedating) antihistamines that have reduced anticholinergic side effects have no effect on colds. Sedation is a major side effect of the first-generation antihistamines and can be incapacitating for some individuals. Ipratropium bromide is a topical anticholinergic agent that is marketed for treatment of rhinorrhea and has effects comparable to those of the first-generation antihistamines. Nasal irritation and occasional bloody nasal mucus are side effects.

Cough associated with the common cold is frequently bothersome, but there are no satisfactory treatments for this symptom. Treatment with the antitussives codeine and dextromethorphan has not been shown to have a clinically significant effect on cough in colds (226).

Although some of these symptomatic treatments appear to have beneficial effects on common-cold symptoms, there is no evidence that the use of these treatments will have an impact on complications. The use of symptomatic therapies in children is not recommended given the inability to demonstrate beneficial effects in this population as well as the potential side effects associated with their use. Notably, the FDA has prohibited marketing of combination cough and cold medications for children due to reports of toxicity with overdoses and even in a small number of children receiving standard doses of these medications.

Other Remedies

The lack of specific therapies, concern about the relative risk of symptomatic treatments, and the relatively benign nature of the common cold have produced popular and commercial interests in the use of alternative medicines for treatment of this illness. Many different nonconventional remedies have been promoted, but few have been subjected to rigorous scientific evaluation. Vitamin C and zinc have been studied extensively; however, meta-analyses found no significant effects on either prevention or treatment (227, 228). Furthermore, intranasal zinc was taken off the market for treatment of colds due to association with anosmia (229). Likewise, clinical trials of oral echinacea or topical iota-carrageenan nasal spray have provided no evidence of efficacy against colds (230–232). Probiotics reduced the rate of RV illnesses in a double-blind, placebo-controlled trial involving preterm infants (233), but had no significant benefit when administered to adults who were experimentally inoculated with RV (234). Treatment with topical corticosteroid can lead to prolonged RV shedding but has no clinically significant effects on cold symptoms (235).

Antiviral Treatment

A variety of antiviral approaches to prevent or treat RV infections have been studied, but no antivirals are currently approved for the treatment of RV infections in the United States. The failure of this effort to produce useful antiviral therapies is due, in part, to the fact that RV colds are self-limited and of short duration. As a result, treatments for the common cold must be rapidly effective, inexpensive, and virtually without toxicity or side effects.

Interferons, given as either prophylaxis or treatment for RV infections, have been studied extensively. Recombinant alpha-interferon given intranasally either as seasonal prophylaxis or as contact prophylaxis was effective for prevention of RV colds but caused local side effects and was not effective when given as treatment after onset of symptoms (108, 236, 237). Inhaled IFN-β has been tested as an approach to prevention of virus-induced exacerbations of asthma. Subjects with persistent asthma and a history of exacerbations provoked by colds were randomized to treatment with either nebulized IFN-β or placebo within 24 hours of the onset of cold symptoms (238). Although IFN-β treatment did not reduce asthma symptoms scores in the study population as a whole, exacerbation risk was reduced in participants with more severe asthma. Larger trials are underway to determine whether inhaled IFN-β could be useful for preventing asthma exacerbations induced by colds in patients with more severe asthma.

Capsid-binding agents bind in a pocket below the "canyon" region of the virion act by altering the conformation of the canyon to prevent receptor binding and/or by stabilizing the capsid and preventing uncoating. Compounds exhibiting this mechanism of action include a variety of isoxazole derivatives, flavonoids, pyridazines, and others. Pleconaril was the most extensively studied of these compounds. In large phase 3 clinical trials, an oral formulation of this drug reduced the total duration of colds by about 1 day and

reduced symptom severity by about 19% (239). This drug was not approved for use as a common-cold treatment by the U.S. Food and Drug Administration because of safety concerns related to induction of cytochrome P450 3A isoenzymes. Vapendavir is a newer capsid binding agent that is now being tested for effects on virus-induced exacerbations of asthma (240). Decoy soluble ICAM-1, antibody to ICAM-1, 3C protease inhibitors, interferon inducers, and the benzimidazole derivative enviroxime have all been studied as common-cold treatments, but none of these has yet been developed into an effective product for prevention or treatment of the RV infection.

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Hepatitis A Virus

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Hepatitis A is an acute, self-limiting infection of the liver by hepatitis A virus (HAV), an enterically transmitted, hepatotropic member of the picornavirus family. Although HAV infection may occasionally result in fulminant hepatitis and death, it is not recognized to cause persistent infection or chronic hepatitis, even in severely immunocompromised individuals.

Reports of icteric disease in early Chinese literature and in the writings of Hippocrates may represent hepatitis A, but the disease discussed cannot be distinguished reliably from jaundice due to other causes (1). The earliest documented outbreaks of probable hepatitis A occurred in soldiers in Europe in the 17th and 18th centuries. Hepatitis A has plagued military campaigns throughout history, and many of the earliest terms used to describe the disease, like *kriegsikterus* and *jaunisse des camps*, reflect this close association (2).

Studies of hepatitis before and during World War II clearly established the existence of two distinct infectious forms of the disease, which later came to be known as hepatitis A and hepatitis B (3, 4). Experimental transmission studies defined the major features of hepatitis A: a relatively short incubation period (15 to 49 days), a fecal-oral mode of transmission, and long-lasting immunity that could be passively transferred. An animal model of HAV infection in marmosets was established by 1967 (5). The responsible virus was visualized in fecal extracts from adult volunteers in 1973 using immune electron microscopy (6), a finding that also resulted in a crude, but sensitive, test for antibody to HAV (anti-HAV). Viruslike particles approximately 27 nm in diameter were specifically aggregated by convalescent, but not preinfection, human sera (Fig. 1). The identification of HAV and the demonstration that infection could be transmitted to marmosets and tamarins, and, later, to seronegative chimpanzees ushered in a new era of research on hepatitis A that culminated in the propagation of the virus in cell culture (7), molecular cloning and sequencing of the viral genome (8), and the subsequent development and licensure of safe, effective vaccines (9, 10). Although the intensity of research on hepatitis A declined significantly following the licensure of effective vaccines, renewed interest in the virus has arisen because of its unusual alternative extracellular forms, circulating within the infected host in a quasi-enveloped form (eHAV) completely cloaked in host membranes, but shed in feces as a highly stable, naked, nonenveloped virion (11).

VIROLOGY

Classification

Based on the structure of its capsid and the organization and sequence of its positive-strand RNA genome, HAV is classified as the type species of the genus *Hepatovirus* within the *Picornaviridae*, a large and diverse family of viruses that includes many other agents of medical and veterinary importance. Although HAV shares many features in common with other members of this family, very limited nucleotide sequence relatedness (12) and several attributes specific to HAV (13) distinguish it from other picornaviruses and warrant its classification in a separate genus. Other viruses classified within the hepatoviruses include HAV strains recovered from nonhuman primates and viruses identified recently in bats, hedgehogs, shrews, and rodents, some of which appear to share antigenic determinants with human HAV (14).

Physical Characteristics of HAV

Genome Organization

The single-stranded, messenger-sense RNA genome of HAV contains a single, long, open reading frame (ORF) flanked by both 5′ and 3′ untranslated RNA segments (UTRs) (Fig. 2). It lacks the 5′ m^7G cap structure typical of host mRNAs, and is instead covalently bound at its 5′ end to a virus-encoded protein termed VPg (3B) (15). As with other picornaviruses, VPg likely serves as a protein primer for RNA synthesis. The 5′UTR contains a high degree of secondary and tertiary RNA structure that has been defined by a combination of phylogenetic analyses, functional genetic studies, and direct biophysical and nuclease mapping techniques (16). This part of the genome contains both essential RNA replication elements and a highly structured segment that functions as an "internal ribosome entry site" (IRES) directing interactions of the RNA with 40S ribosomal subunits. The IRES drives internal initiation of viral translation in a 5′ cap-independent fashion, bypassing multiple upstream AUG codons (16). Translation may initiate at either of two AUG codons at positions 735–737 and 741–743, although the second of these codons is preferred (17). Although cap independent, HAV translation requires most, if not all, eukaryotic translation initiation factors, and results in a polyprotein of approximately 2,227 amino acids residues

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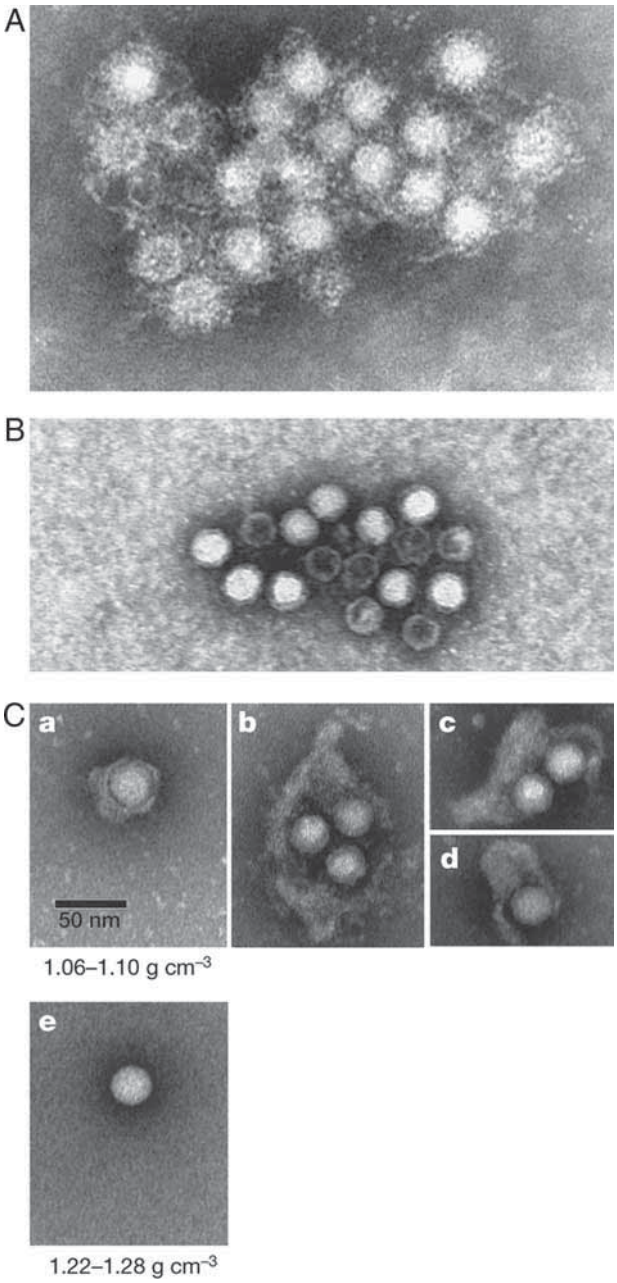

FIGURE 1 Electron micrographs of HAV. (A) Immune electron micrograph of HAV particles from human stool reacted with convalescent serum. The particles are heavily coated with and aggregated by antibody. Both "full" and "empty" particles can be seen. (B) An immune electron micrograph showing particles from human stool reacted with a preinfection serum. The 27- to 28-nm particles are nearly devoid of antibody and some fine structure can be seen. (C) Quasi-enveloped eHAV particles (panels a–d) and a nonenveloped HAV virion purified by density gradient centrifugation from supernatant fluids of infected Huh-7 cell cultures. The density of the fractions containing the particles is shown below the images. (A) and (B) Reprinted from Richman DD, Whitley RJ, Hayden FG (ed) *Clinical Virology*, 3rd ed, with permission. (C) (Reprinted from reference 39 with permission of the publisher.)

that is proteolytically processed into both structural (P1) and nonstructural (2BC and P3) polypeptides (Fig. 2). Following a translation terminator sequence, the genome ends with a 3′ nontranslated region of 63 nucleotides followed by a poly(A) tail of variable length as is typical of picornavirus genomes.

In addition to critical RNA structures within the 5′ and 3′ UTRs that have regulatory functions in the replication cycle, a large, conserved, complex stem-loop structure within the polyprotein-coding segment of the genome (3D region) functions as a *cis*-acting replication element (*cre*) (18). Similar *cre* elements are found in other picornaviruses, although in different regions of the genome, and function as RNA structures (see below) while also encoding proteins to be translated.

Structural and Nonstructural (Replicase) Proteins

The primary cleavage of the polyprotein occurs cotranslationally between the VP1pX and 2B protein segments of the polyprotein, producing P1 (structural proteins) and P2-3 (nonstructural proteins) precursor polypeptides (Fig. 2). This cleavage is mediated by the only protease encoded by the virus, the 3C protein (3C^{pro}) (19, 20). 3C^{pro}, a cysteine protease (21), is responsible for all processing events in the polyprotein with the exception of scission at the VP4-VP2 junction—a late event following RNA packaging into the capsid and that may, in part, be catalyzed by RNA—trimming at theVP1-pX junction, mediated by one or more unknown host proteases (22, 23).

The P1 segment comprises four structural polypeptides in order from the amino terminus, VP4 (also known as 1A), VP2 (1B), VP3 (1C), and VP1pX (1D), named according to picornaviral convention, with VP1 being the largest (Fig. 2). The carboxy-terminal pX extension is found only in quasi-enveloped eHAV particles, and is absent in the mature, nonenveloped virion shed in feces (see below). Thus, the proteins in the mature naked capsid are approximately 23, 222, 246, and 273 amino acids in length, respectively. While pX is often referred to as "2A," this segment of the polyprotein lacks homology with any other picornaviral 2A proteins (indeed, with any other protein in the database), and does not possess the *cis*-active protease activity found in the 2A proteins of other picornaviruses. pX functions in capsid assembly and is present in early assembly intermediates (24–26), and is thus best considered a part of the structural protein complement of the virus (i.e., P1 segment). VP4 (1A) is substantially smaller than its homologs in other picornaviruses, but recently has been confirmed to be present in the HAV capsid (27).

Each of the nonstructural proteins derived from the P2-P3 segment of the polyprotein is likely to contribute to assembly of a membrane-bound viral replicase complex that is responsible for synthesis of new RNA genomes. Unlike other *Picornaviridae*, HAV has no nonstructural 2A protein. 2B and 2C, and probably the unprocessed precursor 2BC, are involved in directing rearrangements of cellular membranes required for replicase assembly (28, 29). The 2B protein is very hydrophobic, and may anchor the replicase complexes to altered intracellular membranes (28). On the other hand, 2C has NTPase activity and contains a helicase motif. The P3 nonstructural proteins include an RNA-dependent RNA polymerase (3D^{pol}), the cysteine protease (3C^{pro}), and 3B (VPg), the protein primer for RNA synthesis. VPg is likely to be doubly uridylated in a slide-back reaction templated by the RNA *cre* and catalyzed by 3D^{pol} and 3CD (30). It remains covalently linked to the 5′ end of both positive- and negative-strand RNAs, but is probably stripped from the

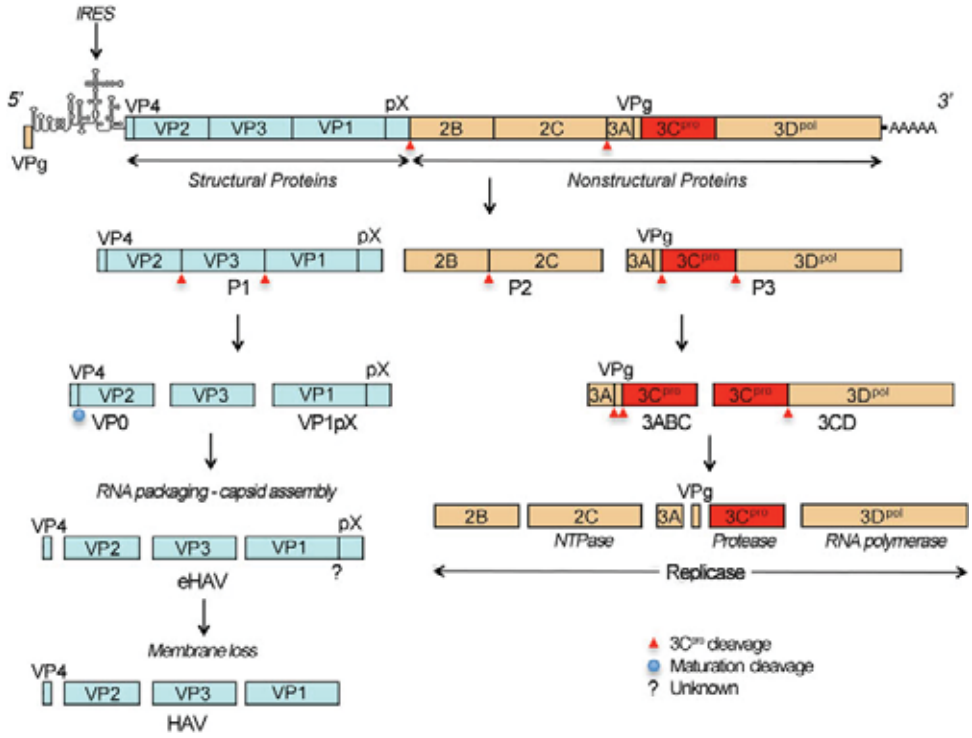

FIGURE 2 HAV genome organization and polyprotein processing cascade. The positive-strand RNA genome of HAV is approximately 7.5 kilobases (kb) in length and contains a single long open reading frame (ORF, box) flanked by relatively short 5′ and 3′ untranslated regions (UTRs, solid lines) as shown. The 5′ UTR is covalently linked at the 5′ end to the genome-linked protein VPg (otherwise known as 3B), and contains a highly structured internal ribosome entry site (IRES) that drives 5′ cap-independent translation of the polyprotein encoded by the ORF. The 3′ UTR terminates in a lengthy poly(A) sequence. The HAV polyprotein is co- and posttranslationally processed by the viral protease $3C^{pro}$ (red box) that cleaves the polyprotein and its derivatives at sites indicated by the red triangles, producing both the structural (P1, turquoise) and nonstructural proteins (P2+P3, tan and red) that go to form the capsid and replicase complex, respectively. Yet-to-be-identified protease activities cause a final maturation cleavage in the VP0 (VP4+VP2) capsid protein, and trim the C-terminal pX domain off of VP1, during late stages in viral maturation.

positive-strand RNA by an unknown VPg unlinkase following viral entry and release of the genome into the cytoplasm. 3A contains a hydrophobic 21 amino acid stretch that is believed to anchor the 3ABC precursor of VPg to cellular membranes. Interestingly, the 3A transmembrane domain targets 3ABC to mitochondrial membranes, where it proteolytically cleaves mitochondrial antiviral signaling protein (MAVS), an important adaptor protein involved in the induction of interferon responses to virus infection (31).

Virion Structure

The viral genome is encapsidated within a stable icosahedral protein shell (the capsid) comprised of 60 copies of each of the four P1 polypeptides: VP1, VP2, VP3, and VP4. Mature virions purified from the feces of infected humans or chimpanzees band at 1.32 to 1.34 g/cm^3 in cesium chloride (CsCl) and sediment at approximately 160 S (32). Particles with lower density that band at about 1.27 g/cm^3 in CsCl and sediment at 70 to 80 S can often be detected in HAV preparations, and may represent empty capsids devoid of genomic RNA (32, 33) (Fig. 1). Unlike poliovirus and other well-studied picornaviruses, the smallest of the structural proteins, VP4, is not myristoylated at its amino terminus (34). Mutational studies suggest a noncanonical capsid assembly pathway that differs from that of other well-studied picornaviruses, with the 8-kDa carboxy-terminal pX extension on the largest capsid protein, VP1, playing a critical role in assembly of pentamer subunits rather than VP4 as is the case with other picornaviruses (24, 25).

Recent X-ray crystallographic studies of formalin-inactivated virus (27) (Fig. 3) indicate that the surface of the capsid is relatively smooth, devoid of depressions present in other picornaviruses that serve to shield receptor-binding sites from antibodies. The protein chains within the capsid are organized like other viruses in the picornavirus family with the exception of VP2, the amino terminus of which is dramatically repositioned such that it interacts with adjacent pentamer subunits, potentially contributing to the high physical stability of the particle (see below). This "domain swap" recapitulates the capsid structure of insect cripaviruses that are distantly related to the picornaviruses, distinguishing HAV from other mammalian picornaviruses and suggesting an ancient evolutionary relationship (Fig. 3) (27).

Antigenic Composition

HAV strains recovered from humans and from nonhuman primates appear to comprise only a single serotype worldwide (35), a fact that has important implications for the success of vaccines. However, neutralizing murine monoclonal antibodies (MAbs) do not recognize denatured capsid proteins, but only their completely folded native conformations within the capsid. Antisera raised to synthetic peptides or proteins expressed from recombinant DNA show only weak reactivity with native capsids and have very limited virus

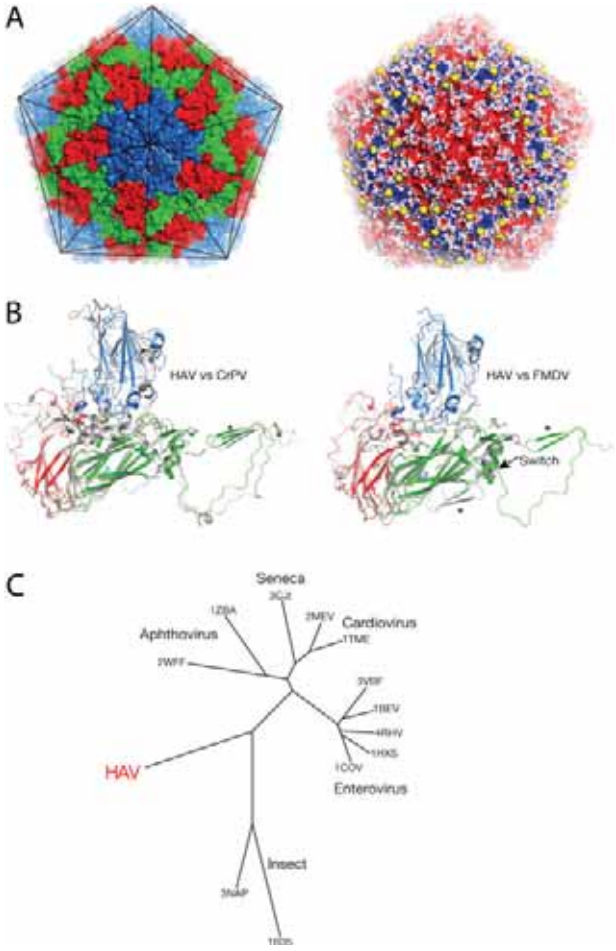

FIGURE 3 X-ray crystallographic structure of the HAV capsid. Overall structure of the HAV capsid as deduced from X-ray crystallographic studies of formalin-inactivated vaccine virus (27). (A) Left: Accessible surface of the capsid, showing residues contributed by VP1 as red, VP2 green, and VP3 blue (60 copies of each are present within one capsid). VP4 is internal and is not seen. Right: Electrostatic surface of the capsid; red=negative charge, blue=positive charge, white=neutral. Yellow dots are sulfate ions. (B) Overlay of folded VP2 structure of HAV with that of VP2 protein from (left) cricket paralysis virus (CrPV) and (right) the mammalian picornavirus, foot-and-mouth virus (FMDV), showing prominent domain-swap paralleling structure in the insect virus. (C) Structural phylogeny of the HAV capsid showing its intermediate position between that of other mammalian picornaviruses and distantly related viruses such as CrPV. (Reprinted from reference 27 with permission of the publisher.)

neutralization activity (36), ruling out the development of vaccines based on recombinant DNA technology.

Antigenic variants of HAV resistant to neutralizing MAbs have been selected by repeated passage of cell culture–adapted virus in the presence of these antibodies (37). These neutralization escape variants contain a limited set of substitutions in closely spaced neutralization epitopes in polypeptide loops within VP3 and VP1 (38). The critical neutralization epitopes of HAV are thus conformationally defined structures rather than linear epitopes, and involve residues of VP1 and VP3. The exact structure of these epitopes is now known from X-ray crystallographic studies (27). Competition studies with various neutralizing MAbs suggest the existence of a single immunodominant site. Remarkably, a combination of only two murine MAbs is capable of efficiently blocking the binding of antibodies present in polyclonal human convalescent sera (38).

Quasi-enveloped eHAV Virions

In addition to the nonenveloped, naked virions described above, HAV has been recognized recently to be released from cells noncytolytically, completely wrapped in host-cell membranes (39) (Fig. 1). The membranes enveloping the capsid in these particles appear to lack any virally encoded protein, distinguishing these "quasi-enveloped" virions (termed "eHAV") from classic enveloped viruses that possess glycosylated viral peplomers on their surface (11). Nevertheless, these eHAV virions are fully infectious in cell culture, yet completely resistant to neutralizing antibodies targeting the capsid in standard infection focus-reduction assays due to the surrounding membranes. The biogenesis of eHAV appears to involve the recruitment of fully assembled, intracellular capsids by components of the cellular endosomal complex required for sorting (ESCRT) complex, and likely involves the budding of the capsid into multivesicular bodies (MVBs) that subsequently release their contents into the extracellular space at the plasma membrane (39) (Fig. 4). This process provides a mechanism for viral egress from the cell, allowing the virus to cross the plasma membrane without cell lysis, and may have many features in common with the biogenesis of exosomes.

Quasi-enveloped eHAV virions are readily separated from naked virions by isopycnic ultracentrifugation in density gradients. The capsid proteins present in eHAV differ from those in the naked virion in that VP1pX is incompletely processed, retaining its 8 kDa carboxy-terminal pX extension (39). pX is clipped off the particle following removal of the membrane with detergents.

Surprisingly, quasi-enveloped eHAV particles appear to be the only form of virus circulating in blood during acute infection, both in humans and in experimentally infected chimpanzees, whereas virus shed in stool is composed exclusively of naked, nonenveloped virions (39). It is likely that both types of virus are produced within hepatocytes, and that the eHAV membrane is lost during passage through the biliary system due to high concentrations of bile salts in the proximal biliary canaliculus. Interestingly, hepatitis E virus, an unrelated positive-strand RNA virus that also causes acute hepatitis, has evolved very similar alternative extracellular forms (11).

Genetic and Antigenic Diversity

Partial sequencing of the genomes of HAV strains recovered from human or nonhuman primate sources in widely separated geographical areas has revealed only limited genomic diversity (40, 41). Thus, primate-derived viruses are closely related genetically, especially when compared with the genetic diversity evident among other picornaviruses. Two major genotypes (I and III) have been described among these strains, as well as two minor genotypes (II and VII), whose nucleotide sequences differ from each other at 15% to 25% of base positions in the genomic region studied (VP1-2A junction). Three other genotypes (IV, V, and VI) each include a single simian HAV strain (41). Much greater diversity is evident among related viruses in bats, shrews, hedgehogs, and rodents, which show a 32.4% to 47.4% distance from human HAV in their amino acid sequences (14). These novel viruses have yet to be assigned to specific

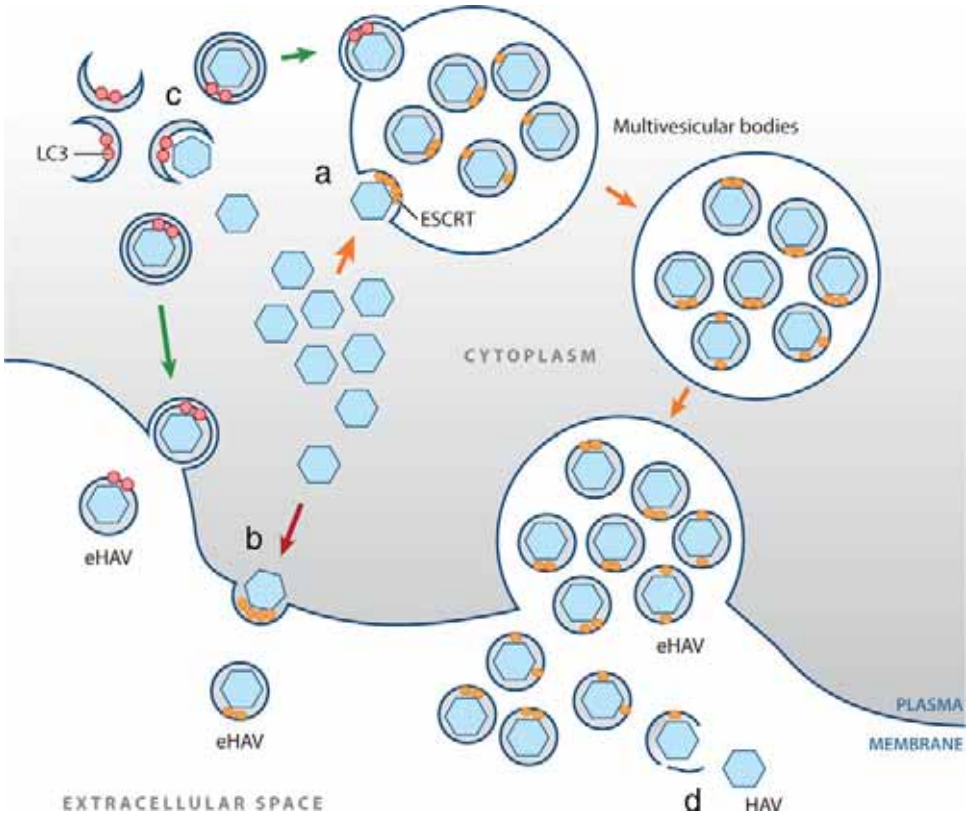

FIGURE 4 Biogenesis of quasi-enveloped eHAV virions (11). Several hypothetical mechanisms may account for the release of quasi-enveloped virions from hepatocytes. The most likely mechanism for eHAV biogenesis involves (a) the recruitment of assembled intracellular HAV capsids to cytoplasmic multivesicular bodies (MVBs) by protein components of the cellular ESCRT (Endosomal Sorting Complex Required for Transport) system such as ALIX, followed by the budding of capsids into MVBs such that they become enclosed in membranes within the MVB. Movement of the MVB to the plasma membrane and fusion of the outer MVB membrane and plasma membrane then delivers eHAV to the extracellular environment. Alternatively, (b) ESCRT-associated proteins might mediate release of eHAV directly at the plasma membrane. A third possibility (c) is that HAV capsids are engulfed in autophagosomes for transport to either MVBs or the plasma membrane. Loss of the eHAV membrane after egress from the cell (d) leads to the production of naked, nonenveloped virions. (Reprinted from reference 11 with permission of the publisher.)

genotypes, but they are close enough in sequence to human HAV to warrant their assignment to the *Hepatovirus* genus. A considerable number of distinct HAV strains have been entirely or nearly entirely sequenced (8, 14, 42–44). Multiple, cell culture–adapted variants derived from one human HAV strain (HM175) have also been fully sequenced, revealing genomic regions that undergo change during propagation in cell culture and may result in attenuation of the virus (42, 45–47).

HAV strains recovered from humans demonstrate high-level (90% to 95%) conservation in the amino acid sequences of the viral capsid proteins. Consistent with this finding, viruses belonging to distinct genotypes elicit antibodies with substantial cross-neutralizing activity, indicating that these viruses comprise only a single HAV serotype (35). Although some MAbs are capable of distinguishing unique epitopes that are variably present in strains of HAV isolated from humans or from naturally infected cynomolgus and African green monkeys (44, 48), simian and human strains of HAV demonstrate substantial antigenic cross-reactivity. This close antigenic relatedness may extend even to non-primate hepatoviruses, as some bat sera appear to recognize human HAV antigens (14). Given the distance between sequences encoding structural proteins, however, it seems likely that at least some of these recently identified viruses comprise one or more additional HAV serotypes.

Stability and Resistance to Chemical Agents

In common with type C enteroviruses, the naked, nonenveloped HAV particle is stable at low pH (pH < 3.0) (49, 50). However, the thermal stability of HAV is considerably greater than that of enteroviruses (50, 51). Incubation of the virus for 4 weeks at room temperature results in only a 100-fold decrease in infectivity. Significant loss of infectivity starts to occur with exposure at 60°C for short periods and infectivity is destroyed almost instantaneously by heating above 90°C (52). However, outbreaks of hepatitis A have been reported following ingestion of partially cooked shellfish, suggesting that brief steaming may be insufficient to destroy the virus. In addition, HAV infectivity is highly resistant to drying, and infectious virus has been recovered from acetone-fixed cell sheets. It is also highly resistant to detergents, surviving a 1% concentration of sodium dodecyl sulfate, as well as to such organic solvents as diethyl ether, chloroform, and trichlorotrifluoroethane (50, 53). Solvent-detergent inactivation procedures thus do not reduce the infectivity of HAV, explaining why hepatitis A transmission has occasionally been associated with the administration of

high-purity clotting-factor concentrates that are devoid of antibody (54). These properties of the virus may contribute significantly to its ability to persist in the environment and cause common-source outbreaks.

HAV can be reliably inactivated by autoclaving (121°C for 30 minutes) and by exposure to hypochlorite (chlorine bleach) in concentrations of 1.5 to 2.5 mg/l for 15 minutes (53). Although chlorine is most commonly used to avoid HAV contamination in water, environmental surfaces can also be decontaminated by quaternary ammonium formulation containing 23% hydrochloric acid (toilet-bowl cleaner). Glutaraldehyde (0.50% for 3 minutes), iodine (3 mg/l for 5 minutes) and potassium permanganate (30 mg/l for 5 minutes) probably are also effective. HAV is also inactivated by short incubation (5 minutes at 25°C) in 3% formalin or in diluted formalin for 3 days at 37°C, and by ultraviolet irradiation (55, 56).

Biology of HAV

Host Range

Serologic studies and direct experimental challenge support the capacity of human HAV strains to infect chimpanzees and other old world primates, including vervet, rhesus, and cynomolgus monkeys, as well as several species of new world primates, including tamarins (*Saguinus* sp.), marmosets (*Callithrix* sp.), and squirrel (*Saimiri* sp.) and owl (*Aotus* sp.) monkeys. Chimpanzees have extensively been used as a model of human HAV infection (57, 58), as have marmosets (59) and owl monkeys (60). HAV has also been isolated from monkeys in the wild. Some simian strains have significant sequence variation and minor antigenic differences with human HAV (44, 61). Guinea pigs are susceptible to experimental infection, but replication is very limited and there is no pathology (62). Nonetheless, a much broader host range for hepatoviruses is indicated by the recent discovery of multiple HAV-like viruses among bats, shrews, hedgehogs, and rodents (14). Whether any are capable of infecting humans or nonhuman primates is not known.

Growth in Cell Culture

HAV was first isolated *ex vivo* in marmoset liver explant cultures and was subsequently propagated in continuous fetal rhesus monkey kidney cells (7, 63). HAV can be propagated in a variety of different types of mammalian cells, including those of primate origin such as BS-C-1, FRhK-4, and MRC-5 cells (64, 65). However, wild-type viral strains from infected patients usually replicate very slowly and to relatively low titers in cultured cells, requiring weeks to months to reach maximal titers. With continued *in vitro* passage, the virus becomes progressively adapted to growth in cell culture, replicating more rapidly and achieving higher titers (65). Cell cultures of murine, guinea pig, porcine, or dolphin origin can also support HAV growth (66).

In contrast to the invariably transient nature of HAV infections in humans, infection of cultured cells is typically noncytopathic and commonly leads to long-term persistence of the virus in cells. This is consistent with the fact that HAV replication does not induce shutoff of cellular protein or nucleic acid synthesis as observed with poliovirus. However, highly cell culture–adapted variants of HAV that replicate very rapidly can cause cytopathic effects in culture, and can even be adapted to conventional plaque assays (47, 67). Cellular injury appears to arise from the induction of apoptotic pathways leading to programmed cell death (67). Continuous passage of the virus in cell culture may result in a reduction in the ability of the virus to replicate and cause disease in primates (68). Adaptive mutations that permit HAV to replicate efficiently in cell culture include mutations within the IRES that enhance cap-independent viral translation in a cell-type–specific fashion and mutations within 2B that promote viral RNA replication in multiple cell types (69–72).

As with other positive-strand viruses, purified genomic RNA, whether extracted from virions or produced synthetically from cloned cDNA, is replication competent when transfected into permissive cultured cells (73). This characteristic has allowed for reverse molecular genetics studies that have elucidated many aspects of HAV biology. However, recovery of virus from synthetic wild-type RNA (in contrast to HAV RNA with cell-culture–adaptation mutations) is difficult in transfected cell cultures, and usually requires inoculation into the liver of susceptible primates (74).

Viral Attachment and Cellular Entry

HAV enters cultured cells via two distinct mechanisms. Quasi-enveloped eHAV particles enter via an acidification-dependent endosomal pathway that entails very slow uncoating of the viral RNA, probably subsequent to dissolution of the enveloping membranes in a late endosomal/lysosomal compartment (39). Initial attachment is likely mediated through phosphatidylserine receptors, as the uptake of eHAV by plasmacytoid dendritic cells is reduced in the presence of annexin V (75). In contrast, entry of mature, nonenveloped virions occurs rapidly and is not inhibited by agents blocking endosomal acidification. A specific cellular protein, HAVCR1 (also known as TIM-1), a mucin-like glycoprotein, has been suggested to serve as a receptor for the virus (76, 77). HAV binds to the cysteine-rich, globular C-terminal extracellular domain of the protein (78). TIM-family proteins facilitate the entry of many enveloped viruses by mediating interactions with phosphatidylserine on the virion surface, suggesting a possible role in quasi-enveloped eHAV entry (79). However, HAVCR1 is widely distributed in different tissues, and the hepatotropic nature of HAV infection cannot be explained by this interaction. Although the hepatocellular asialoglycoprotein receptor has also been suggested to play a role in viral entry by mediating the uptake of IgA-virus complexes (80), it cannot explain initial infection of the liver before the development of antibodies. X-ray crystallography has revealed that the HAV capsid lacks a receptor interaction site similar to those found in other picornaviruses, and this finding has led to speculation that HAV may have a completely different mechanism of cellular entry (27). More studies are needed to define this aspect of the viral replication cycle.

Translation and Replication of the HAV Genome

Following viral entry and release of the genome into the cytoplasm, the RNA undergoes translation under direction of the IRES, leading to expression of both structural and nonstructural proteins (Fig. 5). The nonstructural proteins (2BC) direct the reorganization of intracellular membranes into a tubular-vesicular membranous network within which they direct the synthesis of new viral RNAs (28, 29). The HAV IRES requires intact cellular initiation factor eIF-4G to function, which distinguishes it from other picornavirus IRES (81). Several cellular proteins, including polypyrimidine track-binding protein (PTB), significantly stimulate its ability to direct internal initiation of translation (82). Studies of other picornaviruses suggest that the virion RNA serves as a template for negative-strand RNA synthesis by the RNA-dependent RNA polymerase $3D^{pol}$, thereby

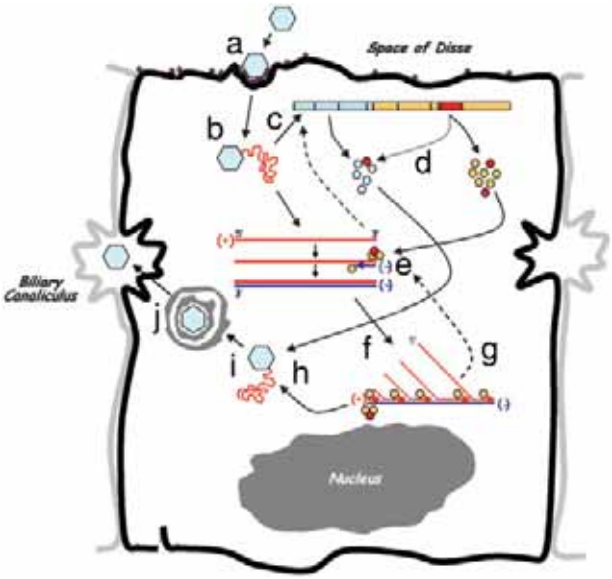

FIGURE 5 HAV replication cycle. (a) HAV interacts with a cellular receptor (possibly HAVCR1/TIM-1) at the basolateral membrane of the hepatocyte, is internalized, and (b) the viral genome is released into the cytoplasm; (b) the positive-strand RNA genome is translated in a cap-independent IRES-driven manner, resulting in a polyprotein that is (d) proteolytically processed to generate nonstructural proteins involved in genome replication (2B, 2C, 3AB, $3D^{pol}$) and the protease ($3C^{pro}$), as well as capsid proteins (see Fig. 2). Changes in intracellular membranes are induced by 2BC, resulting in assembly of the nonstructural proteins into a membrane-bound replicase complex that (e) directs the synthesis of a complementary minus-strand RNA intermediate (blue) that is then used as template to (f) generate multiple new copies of positive-strand RNA (red). These newly synthesized positive-strand RNAs can (g) be directed to engage in additional translation or RNA synthesis or (h) packaged into capsids to generate intracellular viral progeny. These newly assembled viral particles (i) are recruited to multivesicular bodies for ultimate release from the infected cells across either (j) the apical plasma membrane into the biliary canaliculus (as shown) or across the basolateral membrane into the hepatic sinusoids (not shown). (Adapted from Martin A, Lemon SM, *Hepatology* 2006 with permission of the publisher.)

generating a fully double-stranded duplex, which acts in turn to template the synthesis of multiple positive-strand RNA progeny (Fig. 5). As with poliovirus, 3B (VPg), the small genome-linked protein, is thought to function as a protein primer for viral RNA synthesis following its uridylation by $3C^{pro}$ and 3CD in a reaction templated by the *cre* (18).

Virion Assembly and Release

Assembly of the HAV capsid differs significantly from the process followed by other picornaviruses. The C-terminal pX extension of VP1 is essential for the P1 capsid protein precursor to fold as required for efficient $3C^{pro}$ processing and for assembly of pentamer subunits that contain five copies each of VP4-VP2, VP3, and VP1pX (24, 25). Twelve of these pentamers subsequently assemble into a complete capsid, thereby packaging newly synthesized RNA genomes and triggering the cleavage of VP4-2 to VP4 and VP2. This "maturation cleavage" is likely associated with a conformational rearrangement of the capsid proteins that stabilizes the final structure. Current data suggest that these nascent VP1pX-containing virions are then recruited to MVBs through interactions with ALIX and probably other ESCRT-related proteins, acquiring a membrane as they bud into this compartment (39) (Fig. 4). Fusion of the outer MVB membrane with the plasma membrane then releases membrane-wrapped eHAV virions to the extracellular environment.

Several lines of evidence, including unpublished data from our laboratory, suggest that release occurs across both the apical (canalicular) and basolateral (liver sinusoidal) membranes of polarized hepatocytes (83, 84), with loss of the eHAV membrane mediated by high bile-salt concentrations in the proximal biliary canaliculus. The pX domain of VP1pX is subsequently trimmed off the particle, resulting in fecal shedding of naked HAV virions produced in the liver and secreted via the biliary tract, coupled with a viremia composed of quasi-enveloped eHAV virions. Large numbers of naked virions have been visualized in the bile of infected chimpanzees (85), supporting this scenario. However, an enteric site of replication for fecally shed virions cannot be ruled out.

PATHOGENESIS

Animal Models

Current understanding of the pathogenesis of hepatitis A comes largely from studies of experimentally challenged nonhuman primates. Many of these studies were carried out several decades ago and used currently outdated methods for detecting virus and assessing immune responses. However, recent detailed analyses describe HAV infection in two intravenously inoculated chimpanzees, and a third, cohoused animal that became infected by the natural fecal-oral route (57, 58, 75) (Fig. 6). Infection is self-limited in chimpanzees and other nonhuman primates and is generally similar to nonicteric infections in children rather than more severe presentations of hepatitis A in adults. Following inoculation intravenously or orally, a lengthy incubation period (2 to 5 weeks) is followed by the relatively abrupt onset of liver injury marked by elevations of ALT and inflammatory infiltrates within the liver. In tamarins, the duration of the incubation period (measured to the onset of elevated serum liver enzyme activity) correlates inversely with inoculum size, increasing by approximately 5 days for each $\log_{10}$ reduction in dose (86). Progressively higher amounts of virus are found in serum and shed in feces throughout this phase of the infection (87). Both fecal shedding and the magnitude of the viremia begin to decline abruptly following the elevation of serum alanine aminotransferase (ALT), a measure of liver injury. Virus-specific antibodies first appear at this point in the infection (Fig. 6), which typically occurs several weeks after challenge. Laboratory parameters return slowly to normal over a period of 4–12 weeks, although, for reasons that are not understood, viral genomes persist within the liver in slowly decreasing quantities for months after infection in chimpanzees (57). It is not known whether the presence of this RNA indicates the persistence of infectious virus.

Tissue Tropism

Liver

While there may be extrahepatic sites of replication of HAV (discussed below), pathology for the most part is restricted to the liver. HAV, like many other picornaviruses, is very organ specific, perhaps because of specific hepatocyte receptors or intracellular replication factors. Large amounts of virus are

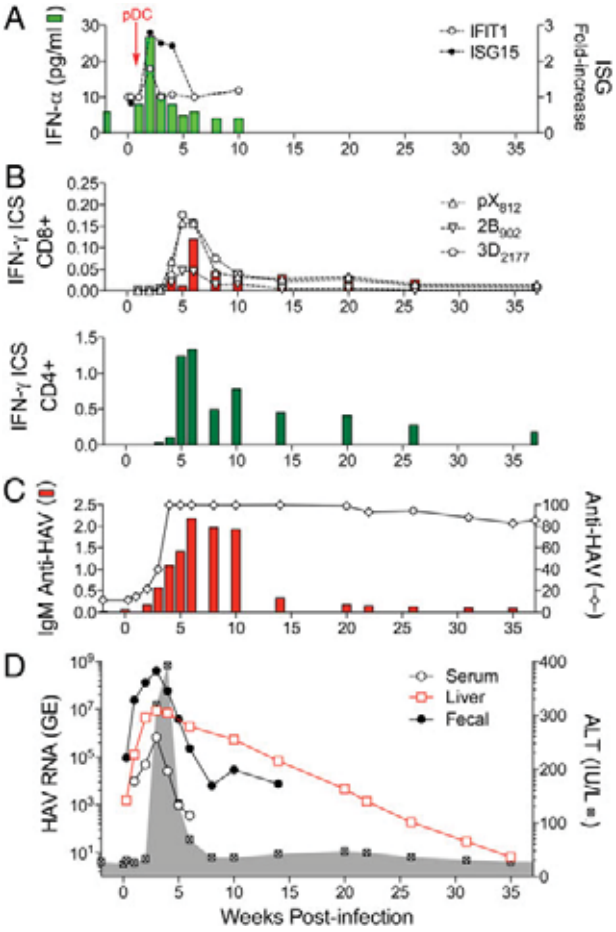

FIGURE 6 Composite graph depicting HAV infection in an intravenously inoculated, experimentally infected chimpanzee (105). (A) Innate immune response: minimal increases in serum IFN-α occur during the first weeks of the infection in association with the transient presence of plasmacytoid dendritic cells (pDCs) in liver sinusoids and minimal intrahepatic expression of interferon-stimulated genes (ISGs) such as IFIT1 and ISG15. These early responses diminish prior to the peak in viral replication. (B) Intracellular cytokine staining shows that the virus-specific CD4+ T cell response (bottom) is more robust than the virus-specific CD8+ T cell cytokine or tetramer-specific response (top), and that the CD4+ T cell response correlates better with viral control. (C) IgM and total anti-HAV antibody responses detected in enzyme-linked immunosorbent assays. The total anti-HAV is measured in a blocking assay, where 100% indicates high-titer antibody. (D) Relative viral RNA abundance (GE=genome equivalents) in serum, liver tissue, and feces determined by reverse transcription polymerase chain reaction assays. Results are layered onto serum alanine transferase values that show a sharp spike indicative of acute liver injury at 4 weeks post inoculation. (Reprinted from reference 105 with permission of the publisher.)

present within the liver throughout the asymptomatic, prodromal, and acute phases of the infection (Fig. 6). Viral antigen has been identified by immunofluorescent microscopy and immunohistochemistry within hepatocytes and in tissue-resident macrophages (Kupffer cells) of experimentally infected nonhuman primates (88), whereas electron microscopy has revealed viruslike particles enclosed within membrane-limited vesicles in hepatocytes (89) (Fig. 7).

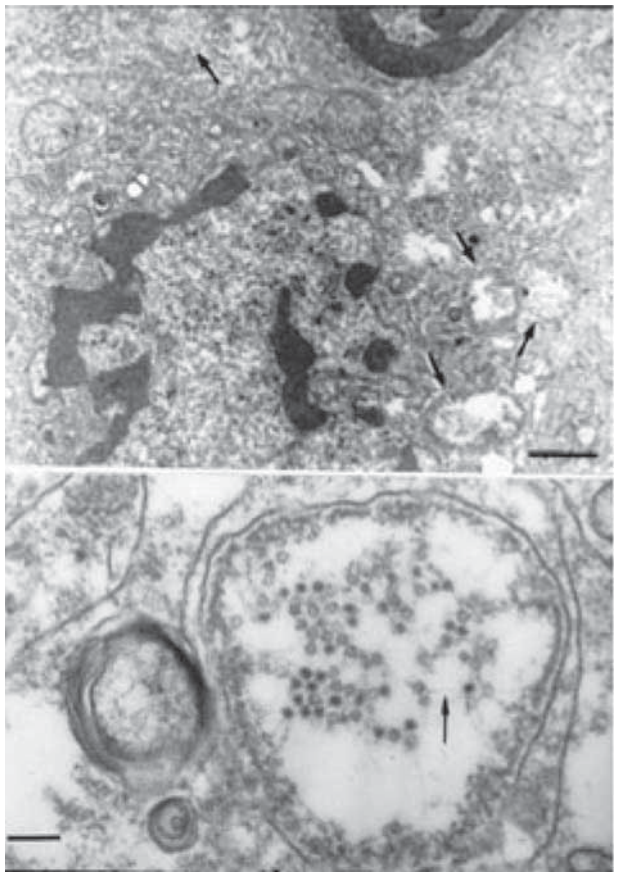

FIGURE 7 Electron micrographs of a marmoset hepatocyte during acute hepatitis A. The arrows in the upper panel point to cytoplasmic vesicles containing probable HAV capsids. The lower panel is a higher powered view of one of the vesicles more clearly showing viruslike particles. Bar=500 nm in the upper panel and 100 nm in the lower. Courtesy of Yohko Shimizu.

While it is clear that antigen present in hepatocytes is indicative of active replication, antigen found in macrophages may reflect only the scavenger function of these cells.

In chimpanzees, low-grade, focal hepatocellular necrosis is observed in the early stages of the infection in chimpanzees (90), followed by more severe changes as serum ALT activity rises; these changes include increasing focal areas of necrosis in the lobular periphery and then throughout the hepatic parenchyma, coupled with widely scattered Councilman bodies, remnants of apoptotic hepatocytes. Periportal inflammatory cell infiltrates composed of lymphocytes and occasional polymorphonuclear leukocytes are present in the liver of infected chimpanzees and owl monkeys (60) as well as from humans (91) (Fig. 8). Virus particles are abundant in the bile of chimpanzees (85), reflecting secretion of HAV from hepatocytes into the proximal biliary canaliculi—commonly considered to be the source of virus shed in feces. Thus, both the naked virus particles shed in feces as well as quasi-enveloped virus circulating in blood (39) originate from hepatocytes.

Wild-type or low-passage HAV is not cytopathic in cell culture, and liver histopathology does not suggest widespread necrosis or apoptosis of hepatocytes in vivo. The presence of large quantities of virus in hepatocytes before the onset of hepatic inflammation and ALT elevation also argues against a

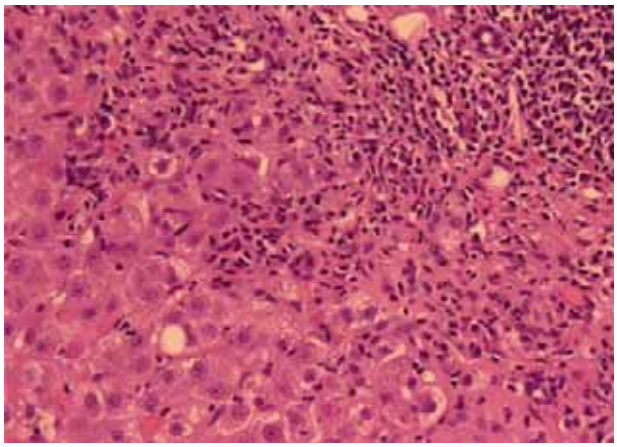

FIGURE 8 Photomicrograph of a liver section from a patient with acute hepatitis A, showing inflammation of the portal and periportal areas by lymphocytes coupled with lobular disarray and hepatocellular ballooning degeneration (cytoplasmic vacuolization). (Hematoxylin and eosin stain; original magnification 40x). (Adapted from Martin A, Lemon SM, *Hepatology* 2006 with permission of the publisher.)

major direct cytopathic effect of HAV. Clinical hepatitis coincides with the appearance of cellular and humoral immune responses, and the pathology of hepatitis A is thus likely to result from the immune response to the infection.

Gastrointestinal Tract

It is not known how HAV reaches the liver in the initial stages of the infection. Because the virus is acid resistant, it can survive passage through the stomach and thus could initially replicate somewhere lower in the gastrointestinal tract, although this hypothesis remains to be proven. While HAV antigen was identified by immunofluorescence microscopy in isolated cells lining the crypts of both the jejunum and ileum in owl monkeys in which the virus was inoculated via feeding tube (60), a similar search for viral antigen in gut tissue failed to identify any infected cells in a cohort of intravenously infected marmosets (88). Virus has also been identified in saliva from infected chimpanzees, but the significance is uncertain (92). The lack of compelling evidence for a primary site of replication in the gut rules out this possibility, however, given similar difficulties in identifying a gastrointestinal site of replication for noroviruses. An alternative possibility is that HAV might be taken up by specialized M cells in the small intestine, undergo transcytosis, and pass into the lymphatics, as proposed for poliovirus (93).

Other Extrahepatic Sites of Replication

Viral antigen has been detected in splenic macrophages and in Kupffer cells, but this finding may not represent active replication in those cells (88). Nonetheless, fluorescent *in situ* hybridization has revealed appreciable amounts of viral RNA in the spleen of bats infected with a nonprimate hepatovirus, suggesting that the HAV may replicate in this organ (14). Replication in other organs appears less likely. Meningoencephalitis and transverse myelitis have been described in association with acute hepatitis A (94, 95), as has acute renal failure in nonfulminant hepatitis A (96, 97). However, no direct evidence suggests replication of the virus in renal or central nervous system tissue. A more interesting possibility is the pancreas: acute pancreatitis sometimes accompanies hepatitis A (98).

Immunity

Innate Immune Response

HAV is a surprisingly stealthy virus in chimpanzees, evoking very little type I interferon (IFN-α/β) production and intrahepatic interferon-stimulated gene (ISG) expression despite replicating to high levels within the liver (57) (Fig. 6). This characteristic distinguishes it sharply from hepatitis C virus (HCV), which induces robust transcriptional upregulation of numerous ISGs in the liver. The basis for this difference is not clear. Stable HAV polyprotein-processing intermediates with cysteine protease activity disrupt signaling pathways by cleaving adaptor proteins required for induction of IFN-α/β responses. Thus, 3ABC (Fig. 2) is directed to mitochondrial membranes by a transmembrane domain in 3A, cleaving MAVS (IPS-1), and thereby disrupting signaling from RIG-I-like receptors (31, 99), whereas 3CD (Fig. 2) cleaves TRIF (TICAM-1), which is essential for Toll-like receptor 3 (TLR3) signaling (100). In both cases, the HAV processing intermediates are more active than mature $3C^{pro}$ in effecting these cleavages, indicating an exquisite adaptation of HAV to the human innate immune response. The unrelated HCV NS3/4A serine protease similarly degrades both of these adaptor proteins. However, the HAV $3C^{pro}$ protease also degrades NEMO (IKKγ) (101), which contributes to NF-κB activation and the induction of type I interferons, whereas NS3/4A does not. HAV also achieves much higher levels of viral protein expression than HCV (57), and this is likely to contribute to its greater capacity to suppress interferon responses in the liver.

Despite the low levels of IFN-α and ISG expressed in HAV-infected chimpanzees, freshly isolated human plasmacytoid dendritic cells (pDCs) are capable of sensing quasi-enveloped eHAV virions released from infected cell cultures, thereby producing IFN-α through a TLR7-dependent mechanism (75). pDCs appear to be recruited to the liver and are found within the sinusoids during the first week of infection in the chimpanzee but, for reasons that are not clear, such cells are no longer detectable within the liver at the onset of hepatic inflammation.

In vitro studies suggest that natural killer cells are capable of recognizing and lysing HAV-infected cells, and thus may contribute to control of the infection (102). Consistent with phenomenon, microarray analyses of liver tissue from acutely infected chimpanzees are indicative of a strong type II IFN-γ response correlating with the onset of liver injury and elevation of serum ALT (57)

T Cell Immunity

The mononuclear inflammatory infiltrates that typify the histopathology of acute hepatitis A (90, 91) have long suggested that adaptive T cell responses may mediate HAV-associated liver injury. Early studies demonstrated that HAV-specific CD8+ cytotoxic T cell clones could be isolated with appropriate cytokine stimulation from the livers of acutely infected humans, whereas CD4+ T cell clones were more likely to be recovered during convalescence (103). The CD8+ T cells produce IFN-γ and are capable of killing HAV-infected fibroblasts in cell culture (103, 104). Granzyme B and perforin mRNA transcripts are also upregulated in acutely infected chimpanzee liver (57). These findings indicate that CD8+ T lymphocytes mediate liver damage and

possibly contribute to viral control. However, recent studies in infected chimpanzees suggest a more important role for noncytolytic virus control mediated by virus-specific CD4+ helper T cells (58, 105). Direct *ex vivo* analyses demonstrated that virus-specific CD4+ T cells are multifunctional, and produce multiple cytokines (IFN-γ, TNF-α, IL-2 and IL-21) in response to a variety of HAV-specific peptides (58). This CD4+ T cell response correlates temporally with reductions in viremia, and the decline in HAV genome copy number in the liver during convalescence (Fig. 6). In contrast, direct *ex vivo* analysis revealed the CD8+ T cell response to be relatively abbreviated, and that virus-specific CD8+ T cells acquire effector functions only after viremia had begun to decline. These findings thus suggest an alternative model of noncytolytic, cytokine-mediated control of the infection (105). T cell responses to HAV infection are thus important for recovery and pathogenesis, but their role in subsequent protection against reinfection is not known.

As discussed above, a member of the T cell immunoglobulin mucin family, HAVCR1 (TIM-1), has been suggested to function as a cellular receptor for HAV (77). This cell surface receptor family is important in T cell regulation. TIM-1 stimulates T cell expansion and cytokine production and is associated with atopic disease (106). An inverse relationship between asthma and childhood exposure to HAV has been suggested but not conclusively demonstrated (107). One hypothesis is that activation of T cells through TIM-1 by HAV or by its natural ligand may affect T cell differentiation and the regulation of Th2-driven allergic inflammatory responses, such that recent reductions in childhood HAV infections may be associated with increases observed in the incidence of atopic diseases (108).

Humoral Immunity

Although the onset of liver damage correlates closely with the appearance of circulating anti-HAV antibodies (Fig. 6), neither antibody-dependent nor complement-mediated cytotoxicity has been demonstrated in hepatitis A (109). While circulating immune complexes containing HAV and HAV-specific antibodies (primarily IgM) have been found during acute infection, immunoglobulin and complement deposits are not found at the sites of liver cell damage (110). Virus-specific antibody responses thus play an uncertain role in the pathogenesis of hepatitis A, but they are likely responsible for solid protection against symptomatic reinfection and for the protection afforded by immunization with formalin-inactivated vaccines (111). Passive immunization with pooled human immune globulins (IGs) results in low levels of circulating antibody that nonetheless provide complete protection against symptomatic infection.

The antibody response to HAV infection is vigorous and long lasting. Both IgM and IgG anti-HAV antibodies are capable of neutralizing HAV infectivity (112, 113); they first appear coincident with the onset of hepatic inflammation and ALT elevation (Fig. 6). Microarray assays show that genes involved in B cell development and the recruitment of B cells to the liver (for example, CXCL13) are transcriptionally upregulated to impressive levels in liver biopsies from infected chimpanzees (57). This transcriptional activation persists for months, and IgG antibodies targeting the viral capsid may comprise as much as 12% to15% of all IgG present in convalescent serum collected several months after infection. Immunity to HAV persists for life, and second infections associated with hepatic disease are unknown.

Although nonenveloped HAV virions are readily neutralized by antibody *in vitro*, quasi-enveloped eHAV virions show no reduction in titer when incubated with neutralizing antibodies (39). This is surprising, given the protective nature of anti-HAV and the fact that only eHAV virions are detectable in the blood during acute infection (39). However, while not well understood, eHAV appears to be susceptible to neutralization within an endocytic compartment following uptake into cells. This is evidenced experimentally by the ability of neutralizing antibodies to inhibit viral replication when added to cultures as late as 4 to 6 hours after adsorption and removal of an eHAV inoculum (39). The antibodies appear to traffic to a late endosomal/lysosomal compartment in which the eHAV membrane is slowly degraded, allowing antibodies to interact with the capsid and neutralize infectivity prior to interactions of the capsid with its receptor. Such postentry neutralization is not observed with naked, nonenveloped virions (39). Whether an IgG receptor is involved in trafficking of the antibody is not known.

While serum antibodies are clearly protective against infection, it has been difficult to judge the role of mucosal immunity because antibodies in saliva or feces either are not detected or are present only at very low levels (114). Individuals with agammaglobulinemia are at risk for particularly severe or persistent infections with other picornaviruses, but this risk has not been described with HAV.

EPIDEMIOLOGY

Global Incidence and Prevalence of HAV

The World Health Organization (WHO) estimates that approximately 1.5 million cases of hepatitis A occur worldwide annually, but the rate of infection is probably ten times higher (115). Although HAV is the most common cause of hepatitis globally, major geographic differences in endemicity exist that are linked to the level of economic development and sanitary conditions.

Three principal patterns of endemicity (high, intermediate, and low) are considered to exist worldwide based on the results of age-specific prevalence of anti-HAV antibodies (Fig. 9). In areas of high endemicity, including underdeveloped regions of Africa, Asia, and South America, HAV is readily transmitted due to poor socioeconomic conditions; the prevalence of anti-HAV reaches 90% in younger adults. Most infections in these areas occur in childhood (before the age of 10 years), many of which are asymptomatic or not recognized as hepatitis. The burden of overt disease is quite low in these areas because the majority of the population has achieved immunity to the disease by adolescence. In areas of intermediate endemicity, only 50% to 60% of adults and 20% to 30% of children have been infected because HAV is not transmitted as readily secondary to improved sanitation. Since there are more adults susceptible to infection, larger outbreaks occur and more persons have symptomatic illness. In areas of low endemicity, such as the United States, Canada, and Western Europe, less than 30% of adults have anti-HAV. When HAV is introduced into such populations, cyclical waves of transmission can occur (116).

Important changes have happened in these three classic patterns of global endemicity over the last few decades (Fig. 10). Improvements in sanitation and socioeconomic development in many previously undeveloped regions has resulted in a transition from a high- to intermediate-endemicity pattern. This "epidemiologic transition" has been associated with a paradoxical increase in disease incidence, as it is associated with a decline in the age-specific prevalence of

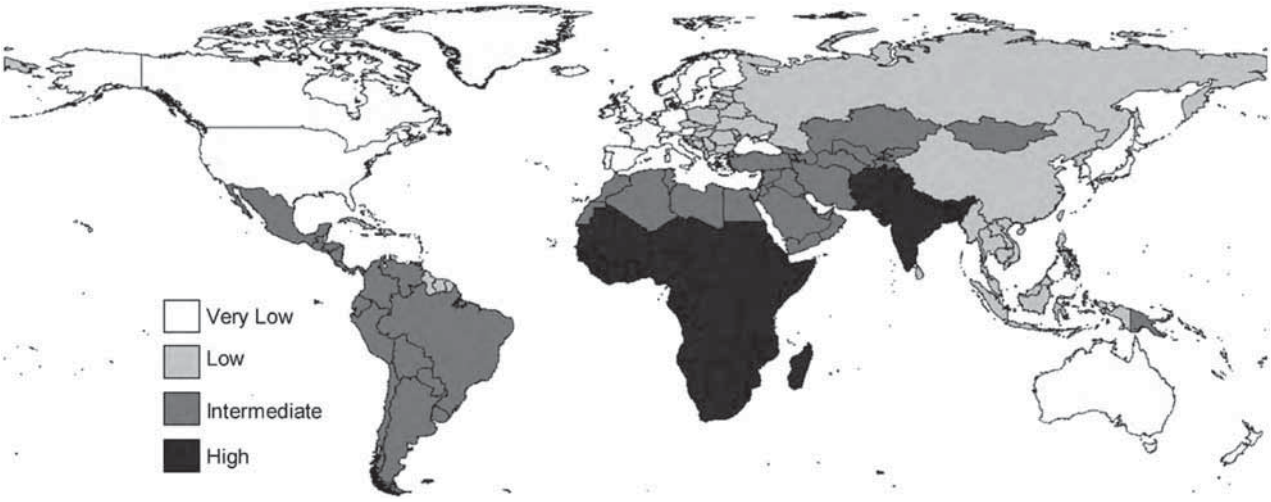

FIGURE 9 Estimated global prevalence of hepatitis A virus, 2005. (Reprinted from reference 117 with permission of the publisher.)

immunity to HAV, leading to increased susceptibility to infection among older individuals, who are more likely to become symptomatic if infected. Regions of transitional endemicity include China and countries in South America, Central and Southeast Asia, and the Middle East (116).

The median age at seroconversion increased between the years 1990 and 2005 (117). This phenomenon can be expected to result in disease patterns characterized by increased morbidity as cohorts of susceptible older children and adults become infected. There is also greater potential for outbreaks as the susceptible population grows, but relatively high levels of circulating virus persist. Significant outbreaks have occurred in China, including one in 1988 caused by the ingestion of raw clams contaminated with HAV, resulting in nearly 300,000 cases (2). Smaller-scale outbreaks have occurred in China more recently, including one in 2006 involving a large school (118). Korea has also recently experienced a large national outbreak of hepatitis A, probably due to the importation of contaminated shellfish from less-developed regions (119). As a result of such outbreaks and increasing morbidity in older populations, more countries are introducing universal childhood immunization programs, the most notable including South Korea and Argentina (120).

Hepatitis A in the United States

The Prevaccine Era

Before licensure of an effective vaccine in 1995, HAV incidence in the United States was primarily cyclic, with peaks occurring every 10 to 15 years. During the 1980s and 1990s, an average of 26,000 hepatitis A cases were reported annually to public health agencies. The outcome of one incidence model, however, predicted an average of 271,000 infections per year between 1980 and 1999, thereby suggesting that only one in ten cases of acute hepatitis A had been reported. More than one-half of those infections, according to the model, were in children aged less than 10 years and likely would have been clinically unrecognizable as hepatitis (121).

Before widespread immunization, approximately one-third of reported cases occurred among children aged less than 15 years, with the highest overall incidence in children

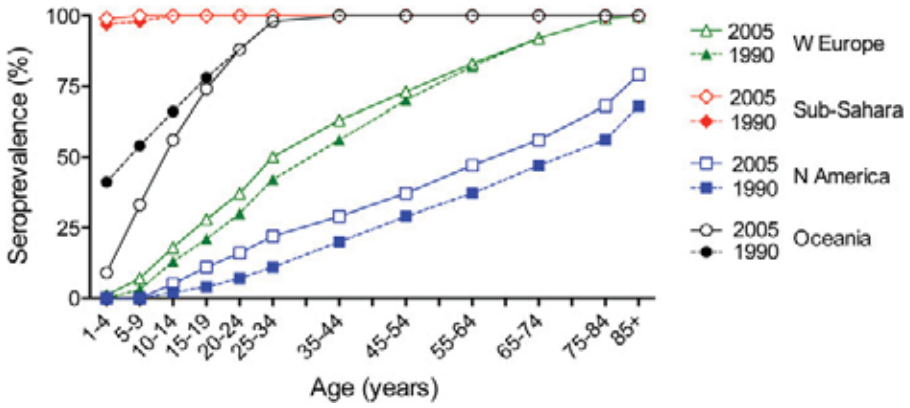

FIGURE 10 Estimates of anti-HAV seroprevalence by age group and selected populations in different world regions, 1990 and 2005. Groups shown include those in Western Europe, South sub-Saharan Africa, North America (high income), and Oceania. The impact of changing standards of sanitation and immunization programs is evident in shifts in age-specific prevalence of anti-HAV antibody. No changes were estimated to have occurred in the African region. (Adapted from reference 117 with permission of the publisher.)

aged 5 to 14 years (122). Large serosurveys conducted between 1988 and 1994 found that the overall prevalence of anti-HAV was 31.3%, with antibody prevalence increasing markedly with age, ranging from 9.4% among persons aged 6 to 11 years to 74.6% among persons older than 70 years. The age-adjusted prevalence was significantly higher among foreign-compared to U.S.-born persons, and was highest among Mexican Americans and lowest among non-Hispanic whites. In a multivariate analysis, only Mexican-American ethnicity and income below the poverty level were associated with HAV infection among U.S.-born children (123).

Nearly half of all hepatitis A cases in the United States were associated with no identifiable source of infection in the prevaccine era (124). Of those cases with an identifiable source, the majority resulted from person-to-person spread of HAV during community-wide outbreaks. Cyclic outbreaks, however, occurred among users of illicit drugs and among men who have sex with men (MSM) (125). Overall, this suggested that nationwide reductions in incidence were more likely to result from routine childhood vaccination than from targeted vaccination of high-risk groups, because children often have unrecognized or asymptomatic infection and play a major role in perpetuating HAV transmission during outbreaks.

Postvaccine Era

The epidemiology of HAV has changed radically since licensure of the hepatitis A vaccine and implementation of a national childhood immunization strategy in the United States. Hepatitis A rates have fallen dramatically and the country has not experienced a nationwide cyclic spike in hepatitis A incidence since 1995 (123). Nevertheless, hepatitis A remains one of the most frequently reported vaccine-preventable diseases in the United States.

Soon after the hepatitis A vaccine was licensed in the United States, the Advisory Committee on Immunization Practices (ACIP) recommended routine vaccination of children aged 2 to 18 years living in communities with the highest rates of infection and disease. An estimated 50% of hepatitis A cases were averted by immunizing children in this age group despite low overall vaccine coverage (10%) (126). Targeting children for immunization resulted in impressive reductions in the incidence of hepatitis A among adults, vividly demonstrating the role young children play in the transmission and propagation of HAV within populations. Although effective regionally, this immunization strategy had only a limited impact on the national incidence of hepatitis A, leading to the expansion of routine immunization to other locations where disease rates were high. By 2003, the incidence of acute hepatitis A had declined overall by 76%, from a rate of 10.7 per 100,000 population during 1990 through 1997 to 2.6 per 100,000 population (127). Due to this precipitous decline, universal vaccination was recommended for all children in the United States aged 12 to 23 months in 2006 (128).

Concurrent with the overall decline in hepatitis A incidence in the United States since the introduction of immunization has been the narrowing of historic differences in rates among racial/ethnic populations and geographical locations. Among Native Americans and Alaska Natives, current rates indicate a 99% decline compared with the prevaccine era and are now approximately the same or less than those of other racial/ethnic populations (129). Rates among Hispanic Americans have fallen almost 90%, although the rates remain higher than those for non-Hispanics (130).

The incidence of HAV-related disease reached the lowest recorded rate (0.4 cases per 100,000 population) in 2011. However, the incidence rate started to increase again in 2012 through 2013, reflecting increased numbers of cases among adults aged more than 20 years. A steady increase in hospitalizations due to HAV infection has been noted since 1999 (131). In 2013, a total of 1,781 cases of hepatitis A were reported from 50 states to the Centers for Disease Control and Prevention (CDC), a 14% increase from 2012 (132). Most cases in the United States are associated with exposure during travel, although nationwide outbreaks still occur due to contaminated food. For example, a nationwide foodborne outbreak in 2013 was found to be associated with a frozen fruit product containing contaminated, imported pomegranate arils (133). Approximately 93% of those infected were adults.

The most recent National Health and Nutrition Examination Survey (NHANES), conducted by the CDC, revealed the prevalence of anti-HAV among adults aged more than 20 years to be 24.2% during 2007 through 2012, a significant decline from 29.5% during 1999 through 2006 (132). The lowest age-specific prevalence was among adults aged 30 to 49 years (16.1% to 17.6%). Thus, a continuing shift in the age-specific prevalence of anti-HAV is occurring subsequent to the institution of universal childhood immunization. This is resulting in a higher anti-HAV prevalence among children but a lower prevalence among adults due to herd protection. The NHANES survey also revealed that vaccination coverage among adults aged 18 to 49 years was only 12.2% in 2012. Although adults are less likely to acquire infection from children during the vaccine era, they are at greater risk of becoming infected through travel and food imported from endemic areas.

Mortality

The overall fatality rate among cases reported through the CDC surveillance system in the United States typically ranges from 0.3% to 0.6%. Between 2009 and 2013, the hepatitis A–related mortality rate was 0.02 deaths/100,000 population per year, which was consistent with rates over the past few decades. Age-specific mortality rates rise with increasing age, ranging from 0.00 deaths/100,000 population among persons aged 0 to 34 years to 0.08 deaths/100,000 population among persons aged 55 to 74 years in 2013, and 0.07 deaths/100,000 population among persons aged more than 75 years (134).

Transmission

The most important mode of HAV transmission is undoubtedly from person to person via the fecal-oral route. Fecal excretion of the virus is highest during the 2 weeks before and a few days immediately after the onset of symptoms but is likely to continue for many weeks (135). The highest infection rates are seen among family and school contacts, indicating that the infective dose is low and interpersonal spread is efficient. The other major mode of transmission is through contaminated food and water. Many types of food products, including seafood, produce, and meat, have been implicated during outbreaks. Transmission following parenteral exposure, including transfusion of blood products and use of contaminated needles, is also possible but is relatively infrequent due to the brief duration of viremia associated with acute infection.

Of the 1,063 cases in the United States in which risk exposure/behavior was reported in 2013, only 24.5% indicated a possible exposure for hepatitis A during the 2 to 6 weeks before the onset of illness. The most frequently identified risk factor for hepatitis A was linkage to a food- or

waterborne outbreak (12.8%). The second most common risk factor was international travel (6.2%), with most cases involving travel to Mexico and Central and South America. Whereas sexual and household contact with another person with hepatitis A have been traditionally among the most frequently identified risk factors, these risk factors were reported for only 5.6% of cases in 2013. MSM and injection drug users accounted for 5.5% and 4.0% of reported cases, respectively (134).

Groups at Increased Risk for Hepatitis A

Travelers

Hepatitis A remains one of the most common vaccine-preventable diseases acquired during international travel. Persons from developed (low-endemicity) countries who travel to developing (intermediate- or high-endemicity) countries are at significant risk for acquiring hepatitis A (136). The risk is higher among travelers visiting areas with poor sanitation and limited access to clean water, although the disease occurs in travelers who report observing strict protective measures and staying in more developed cities or luxury hotels. The incidence rate for nonimmunized travelers is estimated to be 3 cases per 1,000 travelers per month of stay in developing countries. Hepatitis A among Hispanic children who live along the United States–Mexico border has been associated with cross-border travel to Mexico and food-borne exposures during travel (137). Many recommendations and guidelines have been issued in different countries regarding prophylaxis for travelers.

Men Who Have Sex with Men

Numerous outbreaks of hepatitis A have been reported among MSM in the United States, Canada, Europe, and Australia, sometimes in the setting of a larger community-wide outbreak. However, surveys of anti-HAV prevalence among MSM have neither consistently demonstrated a greater propensity for infection compared with a similarly aged population, nor have specific sex practices been consistently identified (138).

Users of Illicit Drugs

Outbreaks among drug users, both injecting and noninjecting, have been reported frequently in the United States and other developed countries since the 1980s (124). In the United States, many outbreaks have involved the use of methamphetamine and transmission by both percutaneous and fecal-oral routes (139). Injection drug users appear to have a higher prevalence of anti-HAV than the general U.S. population (138). Transmission is likely connected to socioeconomic factors, sexual promiscuity, syringe exchange, and contamination of instruments, and to involve both fecal-oral and parenteral routes.

Persons with Clotting-Factor Disorders

Before improvements in viral-inactivation procedures, widespread hepatitis A immunization, and improved donor-screening methods, rare outbreaks of hepatitis A among persons with clotting-factor disorders and hemophilia were reported in Europe and the United States (140)—the result of HAV contamination of high-purity clotting factor concentrates devoid of protective IGs and prepared from very large donor-plasma pools. However, in recent years, no cases of hepatitis A attributable to administration of blood products have been identified in the United States, and hemophiliacs are not at a higher risk than the general population of acquiring HAV (141).

HAV Transmission in Special Settings

Food-Service Establishments

Food-borne hepatitis A outbreaks are recognized relatively infrequently in the United States. According to CDC surveillance in 2013, about 12.8% of cases in which an exposure was reported could be linked to a food-borne outbreak. This was an increase from previous years because of a nationwide outbreak related to contaminated pomegranate seeds in 2013. The proportion of sporadic cases that might be from food-borne sources is unknown but could be considerable, as approximately 50% of reported cases of hepatitis A still do not have an identified source of infection. HAV contamination of agricultural products can occur at any point during cultivation, harvesting, processing, or distribution (142).

A single HAV-infected food handler can transmit the virus to dozens or even hundreds of persons. However, food handlers are not at a higher risk of hepatitis A because of their occupation, and most infected food handlers do not transmit HAV to consumers or restaurant patrons (142).

Molecular epidemiologic techniques comparing RNA sequences of HAV strains has made possible the identification of previously unrecognized links between cases, as exemplified in a multistate outbreak of HAV subgenotype IB infection among European travelers returning from Egypt in 2012 to 2013. A persistent common source of infection was suspected, as HAV strains isolated from various cases over a period of several weeks were indistinguishable genetically. The 107 cases of hepatitis A infection were eventually linked to strawberries (143). Similar molecular epidemiologic studies have elucidated the origins of HAV strains involved in a recent nationwide outbreak of hepatitis A in South Korea (119).

Child Care Centers

The frequency of outbreaks among children attending day care centers and persons employed at these centers has decreased substantially, as the overall hepatitis A incidence among children has declined since the implementation of vaccination, especially in the United States, since 2006. Because infection among children is typically mild or asymptomatic, outbreaks are often not identified until adult contacts become ill (144).

Schools

In the United States, the appearance of cases of hepatitis A in schools is ordinarily a reflection of disease acquisition and transmission in the community. Secondary transmission to other students is uncommon. However, if multiple cases occur among students, a common source of infection is possible and should be investigated (128).

Health Care Institutions

Nosocomial transmission of HAV is rare, and the CDC recommends adherence to standard precautions. Health care personnel who become infected should avoid patient contact and food handling for 7 days after the onset of jaundice. Patients with typical cases of hepatitis A are not routinely admitted to hospitals, and even when patients are hospitalized, the probability of transmission is low because most are admitted after the onset of jaundice, when the period of maximum infectivity has passed. Outbreaks in hospital settings have been linked to inadequate hand cleaning. Anti-HAV seroprevalence is similar in health care workers and control populations, indicating that health care workers are not at increased risk of acquiring infection (145).

Workers Exposed to Sewage

Among wastewater workers, no work-related instances of HAV transmission have been reported in the United States, and serologic surveys have shown no substantial or consistent increase in the prevalence of anti-HAV. Surveys performed in other countries indicate a possible elevated risk for HAV infection among workers exposed to sewage; however, those analyses did not control for other factors, such as socioeconomic status (146).

Nonhuman Primate Colonies

Persons working with captive nonhuman primates were previously at risk for hepatitis A, but immunization of animal handlers has largely eliminated that risk.

CLINICAL FEATURES

The clinical manifestations of hepatitis A are highly variable, ranging from asymptomatic infection, to mild anicteric hepatitis, acute icteric viral hepatitis, and even fulminant hepatic failure. The risk of clinical disease following HAV infection is determined primarily by the age of the person infected. In children younger than 6 years, 70% of infections are asymptomatic and, if illness does occur, it is typically not associated with jaundice. By contrast, infection during late childhood through adulthood is likely to cause icteric illness in more than 70% of patients. The risk of fulminant hepatitis and death is also much higher in older patients.

Uncomplicated Acute Hepatitis A

Clinical Course

Clinical signs and symptoms of acute hepatitis A are indistinguishable from those caused by other types of hepatitis (see Chapter 5 for differential diagnosis). Thus, while clues from the epidemiologic setting may be present, laboratory tests are required for specific diagnosis. The incubation period is approximately 15 to 50 days, with a mean of about 30 days (Fig. 11). In older children and adults, the illness usually begins with abrupt onset of prodromal symptoms including fatigue, malaise, nausea, vomiting, anorexia, fever, and abdominal pain. Typical symptoms of hepatitis, beginning with darkening of the urine and followed by jaundice and pale or clay-colored stools, will appear after a period of several days to a week. During a large shellfish-associated epidemic in Shanghai in 1988, prodromal symptoms included anorexia (82%), malaise (80%), fever (76%), nausea (69%), and vomiting (47%) among the more severely affected patients who were hospitalized (147). Hyperbilirubinemia was seen in 91%, and 84% were overtly jaundiced (147). Itching, often a sign of cholestasis, occurs in less than 5% of symptomatic patients but may be severe enough to require antipruritics and corticosteroid therapy. The two most common physical findings are jaundice and tender hepatomegaly, which occur in 70% and 80% of symptomatic patients, respectively. Less common clinical findings include splenomegaly (9%), rash, arthritis, and leukocytoclastic vasculitis.

Serum ALT and aspartate aminotransferase (AST) activities are sensitive, but nonspecific, measures of parenchymal liver damage associated with acute HAV infection. ALT elevations, usually higher than AST, may be found even during the prodromal stage. In icteric acute hepatitis A, serum ALT levels are typically less than 2,000 IU/l, but may exceed 20,000 IU/l. While high ALT levels occur in patients with severe hepatitis, the elevation of ALT is not necessarily correlated with the severity of the illness. Alkaline phosphatase levels are usually only mildly elevated in hepatitis A, except when the illness is complicated by cholestasis. Biochemical abnormalities may persist for 2 to 3 months, but usually return to normal by 4 weeks (Fig. 11).

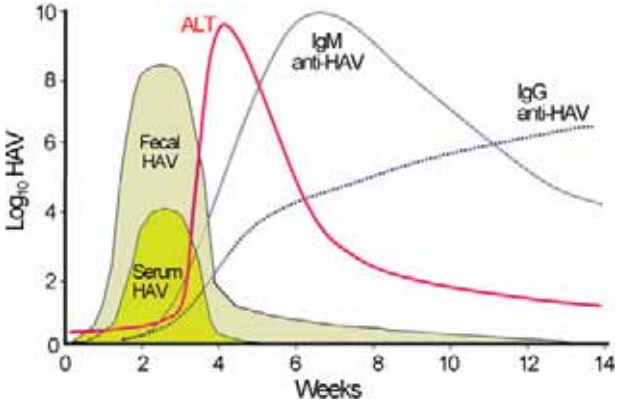

FIGURE 11 Natural history of hepatitis A. The infection is typically acute in nature, with symptoms and signs of the infection usually occurring within 3 to 5 weeks of exposure. The sequence of events includes shedding of infectious HAV in feces and viremia, followed by increases in serum ALT activity (red line), and the appearance of IgM and IgG anti-HAV antibody responses (the latter typically measured as total anti-HAV antibody). (Adapted from Martin A, Lemon SM, *Hepatology* 2006 with permission of the publisher.)

The duration of illness varies. In many patients, the appearance of jaundice is associated with rapid resolution of the prodromal symptoms. After 3 to 4 weeks, most patients feel better, no longer have hepatomegaly, and have normal or near-normal serum levels of ALT and bilirubin. Prolonged jaundice or a relapsing pattern may also occur but ultimate resolution in these cases is universal. Infection with HAV does not cause chronic infection. HAV infection, whether asymptomatic or associated with disease, is associated with the development of a robust immune response, which provides lifelong protection against future reinfection with the virus.

Children

HAV infection in younger children is typically mild; only 30% of children younger than 6 years are symptomatic. Symptoms, when present, are often nonspecific, and include fever, malaise, anorexia, and nausea. Serum aminotransferase levels can be elevated during the prodromal period and jaundice, when it occurs, usually develops occurs 1 to 2 weeks after symptom onset. Jaundice typically lasts for less than 2 weeks, with aminotransferases returning to normal limits in approximately 2 to 3 months. Acute liver failure is extremely rare in children, occurring in less than 1% of cases.

Hepatitis A in Pregnancy

HAV infection in pregnant women is usually self-limiting and not a threat to the fetus. Although HAV infection does not increase the risk of congenital malformations or spontaneous abortions, there have been rare reports of increased preterm labor and premature rupture of membranes (148).

Complications and Atypical Presentations

Cholestatic Hepatitis A

Prolonged jaundice is often associated with fever and pruritus and is an indication of cholestatic hepatitis. Peak serum bilirubin levels may reach 12 to 29 mg/dl, and jaundice may continue for up to 18 weeks (149). The cause of prolonged cholestasis is unknown, but is usually reflected histologically by predominantly cholestatic features. The duration of viremia has been found to be longer in patients with cholestatic hepatitis but the virologic and host factors implicated in the pathogenesis are unknown. In a prospective, Korean multicenter study of 595 hepatitis A cases, prolonged cholestasis occurred in 4.2%; preexisting chronic hepatitis B infection, prolonged prothrombin time, and higher total bilirubin levels were associated with increased risk (150). Peak biochemical markers including serum aminotransferases and alkaline phosphatase were not significantly different in patients with prolonged cholestasis (136). Cholestasis ultimately resolves spontaneously with no complications or sequelae. It is important to be aware of this relatively common but atypical feature of hepatitis A and to avoid overly aggressive interventions.

Relapsing Hepatitis A

A relapsing form of hepatitis A has been observed in 3% to 20% of patients. One study reported 12.5% of 297 adults had a relapsing course, of whom 22% had more than one relapse (151). The relapses are usually milder than the initial illness and typically occur after the serum aminotransferases have normalized from the initial episode, and long after the development of anti-HAV. Nonetheless, it seems likely viral replication is ongoing in such cases: HAV has been found in the stool of some patients during relapse (152). The mechanisms underlying relapse are unknown and predisposing factors have not been identified. Similar minor relapses have been observed in experimentally infected chimpanzees, and were not associated with mutations in the viral capsid that might suggest immune escape. The prognosis for relapsing hepatitis is excellent, and all cases ultimately resolve without chronic sequelae.

Fulminant Hepatitis A

Fulminant hepatitis, characterized by rapid onset of liver failure and coma, is rarely associated with HAV infection but is potentially fatal. Fulminant disease is more common in older persons, and recovery from severe disease is less common in patients older than 50 years. The Acute Liver Failure Study Group (ALFSG) study of adults with acute liver failure from 1998 to 2005 found that HAV accounted for 3.1% of patients and only 0.12% of those listed for liver transplantation (139).

The initial clinical presentation is not significantly different from other cases of acute hepatitis A (153). Patients typically have a coagulopathy (prothrombin time >15 seconds or international normalized ratio >1.5) and encephalopathy. The ALFSG evaluated 29 patients with fulminant hepatitis A and developed a prognostic index to predict transplantation or death. The index incorporates four presenting features (serum ALT <2,600 IU/L, creatinine >2.0 mg/dL, intubation, pressors) and was shown to be better than other published models including the laboratory Model for End-Stage Liver Disease score. Laboratory and clinical evidence of deteriorating liver function correlates with a histologic picture of virtually complete destruction of the hepatic parenchyma with only a reticulin framework and portal tracts remaining. Occasionally, small groups of surviving hepatocytes can be seen close to portal tracts, which may be evidence of regeneration (154). As many as 50% of patients with acute, HAV-associated fulminant liver failure may die or require emergency liver transplantation. Spontaneous recovery rates in fulminant hepatitis A range between 30% and 60%, and survivors regain complete liver function. Prognosis is influenced by age, clotting factor levels, stage of coma, and presence of kidney disease. Recovery from fulminant hepatitis is difficult to predict, and the only effective treatment is liver transplantation.

Host factors associated with increased risk of fulminant hepatitis include older age and underlying chronic liver disease, particularly chronic HCV infection. In a study of 163 patients with chronic hepatitis B and 432 patients with chronic hepatitis C, HAV superinfection occurred in 27 patients (155). All 10 of the patients with hepatitis B infection had an uncomplicated course. In contrast, fulminant hepatic failure developed in 7 of the 17 patients with chronic hepatitis C who acquired hepatitis A, and 6 of those patients died (155). There were 47 deaths (0.015%) recorded among the 310,746 cases in the 1988 Shanghai epidemic that primarily involved adolescents and young adults (147). Of the 47 deaths, 25 were due to fulminant hepatic failure and at least half of the affected individuals had underlying liver disease.

Extrahepatic Manifestations

Rarely, in patients with prolonged illness, extrahepatic disease, including optic neuritis, transverse myelitis, aplastic anemia, and thrombocytopenia, may be noted. Although these conditions are possibly manifestations of immune-complex disease, the relationship of these syndromes to the HAV infection is not established (149). Mild to moderate pancreatitis has also been reported in association with acute hepatitis A (98), but its pathogenesis is equally obscure.

LABORATORY DIAGNOSIS

The serologic detection of IgM anti-HAV is the simplest, least expensive, most sensitive, and most specific approach to laboratory diagnosis. Detection of an HAV-specific antibody of the IgM class (primarily against capsid antigen) indicates current or recent infection and is the gold standard for diagnosis of acute hepatitis A. Such an antibody is almost always present at the onset of symptoms, peaks during the acute or early convalescent phase of the disease, and remains positive for approximately 4 to 6 months (112) (Fig. 11). Many methods have been used to detect IgM anti-HAV, but enzyme-linked immunosorbent assay (ELISA) is now the most commonly used. IgM anti-HAV ELISA assays are available commercially and generally do not detect the low levels of IgM that may persist in patients more than 6 months after acute HAV infection (156).

However, persons who are unlikely to have acute viral hepatitis should not be tested for IgM anti-HAV, and the use of IgM anti-HAV as a screening tool or as part of a test panel used in the workup of nonacute liver function abnormalities should be discouraged. Testing in the absence of clinical signs or symptoms of acute HAV infection lowers the predictive value of the IgM anti-HAV test and can result in increased numbers of false-positive tests for acute HAV infection (157). A positive IgM anti-HAV does not necessarily indicate acute infection because individuals can have a prolonged presence of IgM. In one study of 140 persons reported to have a positive IgM test result in 2003, a total of

87 (62%) did not have an illness that met the case definition for hepatitis A or any other type of viral hepatitis (143).

IgG anti-HAV appears at the same time as IgM antibodies to HAV but, unlike IgM anti-HAV, remains detectable for decades thereafter (Fig. 11). Commercial assays are not generally available for specific detection of IgG anti-HAV. However, multiple types of assays are available for detection of total anti-HAV antibody, which, in the absence of acute infection, is largely comprised of IgG antibody and indicative of previous or resolved HAV infection. Conversely, the absence of anti-HAV in a sample collected during the acute phase of illness or early convalescence is strong evidence against a diagnosis of HAV infection. However, if the clinical or epidemiologic situation strongly suggests HAV infection, the test should be repeated within a few days to a week to formally exclude the diagnosis.

Liver biopsy is rarely indicated to establish a diagnosis of acute hepatitis. This procedure is associated with discomfort to the patient, and carries a small but finite risk of death. Moreover, tissue morphology is usually not diagnostic.

Detection of virus or viral antigen in the stool is a useful research tool but has no place in routine clinical diagnosis. Since HAV clinical isolates usually replicate very slowly and then to very low titers in cell culture, virus isolation is insensitive, unreliable, and expensive.

Nucleic acid detection techniques are more sensitive than immunoassays for viral antigen to detect HAV in samples of different origin (e.g., clinical specimens, environmental samples, or food). Amplification of viral RNA by reverse transcription-polymerase chain reaction (RT-PCR) is currently the most sensitive and widely used method for detection of HAV RNA (44). Real-time RT-PCR is rapid, sensitive, reproducible, and potentially quantitative. Nucleic acid sequencing of PCR amplimers may confirm their specificity and provide the ultimate means of identifying and characterizing the responsible virus genotype or strain (158). Sequencing of selected genomic regions of HAV is used to determine the genetic relatedness of isolates for epidemiologic investigations (159).

RT-PCR may be used for HAV detection in environmental samples. The same characteristics that facilitate the likelihood of transmission of HAV by contaminated food and water—i.e., the stability of HAV in the environment, especially when associated with organic matter, and its resistance to low pH, drying, and heat—also improve the likelihood of detection in environmental samples. However, HAV detection in food traditionally has not been included as a part of outbreak investigations because of the lengthy incubation period of the disease and the probability that the offending foodstuff usually has been consumed or discarded by the time the outbreak is recognized (160).

PREVENTION

General

Hepatitis A is a vaccine-preventable disease. The WHO recommends hepatitis A vaccination be integrated into the childhood schedule if disease incidence and cost effectiveness support its use (115). Before vaccines were licensed beginning in 1995, prevention of hepatitis A was primarily aided by adherence to sanitary practices such as hand washing, appropriate heating of foods, and avoidance of food and water from endemic areas. Hand washing is highly effective in preventing transmission, since the virus can survive for up to 4 hours on the hands (161). General hygienic measures are most important in limiting person-to-person spread in the home, school, or work settings. While nosocomial transmission is rare and hospitalized patients require only enteric precautions and private rooms, gloves should be worn when handling anything that is potentially contaminated (162). Chlorination and household bleach (1:100 dilution) are sufficient to inactivate the virus (see above).

The provision of clean water, availability of proper waste disposal, and general improvement in overall living conditions rapidly reduce the incidence of hepatitis A within a population. However, the epidemiologic transition that occurs as a result of these preventive measures leads to a declining hepatitis A seroprevalence that may pose a public health problem; greater numbers of older people may be susceptible to infection and symptomatic illness.

Passive Immunization

Before the licensure of effective hepatitis A vaccines, IG was the sole means of prevention of hepatitis A for people who either were likely to become infected or had recently been exposed. Passive immunization with polyclonal serum IG prior to exposure has been available since the 1940s and has been shown to decrease the incidence of HAV infection by more than 90% (163). IG is a sterile preparation of concentrated antibodies made from pooled human plasma processed by cold ethanol fractionation (164). In the United States, only plasma that has tested negative for hepatitis B surface antigen, antibody to human immunodeficiency virus, and antibody to HCV is used to produce IG. Since 1995, the U.S. Food and Drug Administration (FDA) has required that the process used to make IG include a specific viral inactivation step or that final products test negative for HCV RNA by PCR (128). Despite concern that the decline in the prevalence of anti-HAV in the population might reduce the effectiveness of IG, there is no standard for anti-HAV levels in IG preparations, and at present no evidence of reduced efficacy of IG.

IG provides protection against hepatitis A through passive transfer of antibody. When administered for pre-exposure prophylaxis, 1 dose of 0.02 ml/kg IM confers protection for no more than 3 months, and 1 dose of 0.06 mL/kg IM confers protection for 3–5 months. When administered within 2 weeks after an exposure to HAV (0.02 mL/kg IM), IG is 80%–90% effective in preventing hepatitis A (163). Efficacy is greatest when IG is administered early in the incubation period. When administered later in the incubation period, IG may only attenuate the clinical manifestations of HAV infection (163).

The level of anti-HAV detected in persons one week after the administration of a single intramuscular (IM) 5-cc dose of IG is typically in the range of 50 to100 mIU/ml (111). By comparison, the titer of anti-HAV detected after recent infection often exceeds 15,000 mIU/ml, and following active immunization with three doses of HAV vaccine, approximately 3,500 mIU/ml. While measurable antibody following IG may disappear rapidly, protection persists for several months (165).

Serious adverse events from IG are rare. Because anaphylaxis has been reported after repeated administration to persons with immunoglobulin A deficiency, those persons should not receive IG. Pregnancy or lactation is not a contraindication to receipt of IG. A thimerosal-free preparation of IG is available and is preferable for use in infants and pregnant women (128). Less-serious reactions can also occur, including pain at the injection site.

Active Immunization

Hepatitis A Vaccines

Several types of hepatitis A vaccine have been developed and evaluated in nonhuman primate models of HAV infection and in human clinical trials. These include formalin-inactivated vaccines (9, 166), live attenuated vaccines (167), and combination HAV and hepatitis B vaccines (168). For the most part, only inactivated HAV vaccines have been evaluated for efficacy in rigorous, prospective, controlled clinical trials, and they comprise the only vaccine type approved for use in the United States (169). However, a live-attenuated HAV vaccine has been used extensively in China since 1992, reportedly with effective results (170). A live-attenuated vaccine (BioVac-A; Wockhardt; Mumbai, India) is also available in India and in other countries (Mevac-A) including Guatemala, Philippines, Bangladesh, Nepal, and Chile.

Havrix (GlaxoSmithKline, Rixensart, Belgium) was the first HAV vaccine licensed for use in the United States in 1995. It is a purified, formalin-inactivated vaccine manufactured from the HM175 virus strain propagated in a human cell line. A second formalin-inactivated vaccine, Vaqta (Merck & Co., Inc., Whitehouse Station, New Jersey), became available in the United States in 1996. It is produced with the CR326 viral strain and has equivalent immunogenicity and tolerance compared to Havrix (171). These two vaccines are indistinguishable in terms of their efficacy. Both have been demonstrated to provide high-level (95% or greater) protection against symptomatic HAV infection in prospective clinical trials of strikingly different design (10, 169). The results of the Vaqta trial even suggested that inactivated HAV vaccine can provide a high level of postexposure protection against disease (10), a fact confirmed in a later clinical trial (172). The dosing and vaccination schedule approved by the Advisory Committee on Immunization Practices is shown in Table 1.

Twinrix (containing both HAV and HBV antigens; GlaxoSmithKline) was approved by the FDA in 2001. Twinrix is licensed for use in persons aged more than 18 years and contains 720 enzyme-linked immunosorbent assay units (EL.U.) of hepatitis A antigen (half the Havrix adult dose) and 20 mcg of recombinant hepatitis B surface antigen protein (the same as the Engerix-B [GlaxoSmithKline] adult dose). Primary immunization consists of three doses according to the same schedule as that commonly used for single-antigen hepatitis B vaccine (0, 1, and 6 months) (Table 2). After three doses of Twinrix, antibody responses to both antigens are equivalent to responses seen after the single-antigen vaccines are administered separately on standard schedules (173). Clinicians may choose to use an accelerated schedule for Twinrix (i.e., doses at days 0, 7, and 21). The FDA approved an accelerated schedule for Twinrix in 2007; persons who receive a vaccination on an accelerated schedule should also receive a booster dose at 1 year after the start of the series to promote long-term immunity (174).

TABLE 1 Recommended regimens: dose and schedule for hepatitis A vaccines

Vaccine	Age (yr)	Dose	Volume (ml)	Two-dose schedule (mo)[a]
Havrix	1–18	720 (EL.U.)	0.5	0 (6–12)
	>18	1,440 (EL.U.)	1.0	0 (6–12)
Vaqta	1–18	25 (U)	0.5	0 (6–18)
	>18	50 (U)	1.0	0 (6–18)

Abbreviations: EL.U.=enzyme-linked immunosorbent assay units;U=units.
[a]0 months represents timing of the initial dose; subsequent numbers represent months after the initial dose.
Adapted from reference 128.

TABLE 2 Licensed dosages of Twinrix

Age (yr)	Dose (hepatitis A/ hepatitis B)	Volume (ml)	No. doses	Schedule (mo)[a]
≥18	720 EL.U.	1.0	3	0, 3, 6

Abbreviations: EL.U.=enzyme-linked immunosorbent assay units.
[a]0 months represents timing of the initial dose; subsequent numbers represent months after the initial dose.
Adapted from reference 128.

Vaccine Immunity

Although inactivated hepatitis A vaccine elicits both anti-HAV antibody and virus-specific T-cell responses (175), protection is likely afforded primarily by serum-neutralizing antibodies, based on early studies with passively transferred, pooled human IG (176). Low levels of neutralizing antibody correlate with high-level protection against disease (111).

Indications for Immunization

Indications for hepatitis A immunization have evolved over time, and the current U.S. recommendation, issued by the Advisory Committee on Immunization Practices (ACIP) of the CDC in 2006, is to routinely immunize all children at 1 year of age. Globally, the WHO recommends immunization for children greater than 1 year of age in places with intermediate to low endemicity (115). These recommendations are intended to further reduce hepatitis A morbidity and mortality and make possible the consideration of eventual elimination of HAV transmission. Immunization is also recommended for persons who are at increased risk for infection and for any person wishing to obtain immunity. Children who are not vaccinated by the age of 2 years can be immunized at later ages. Catch-up vaccination programs are strongly encouraged, especially in the context of increasing incidence and outbreaks since 2011.

Persons at increased risk of infection or more severe disease who should be immunized include individuals traveling to or working in countries with high or intermediate rates of hepatitis A (see specific recommendations below in "Pre- versus Postexposure Prophylaxis"), persons with chronic liver disease (especially chronic hepatitis C) or clotting factor disorders, alcoholics, MSM, illicit drug users, individuals with close personal contact with an international adoptee from a country of high or intermediate endemicity during the first 60 days following arrival to the United States, persons working with HAV-infected primates or with HAV in a research laboratory, and individuals with recent exposure (for postexposure prophylaxis). Immunization against HAV infection is safe and effective in persons with chronic liver disease; susceptible people who are either awaiting or have received liver transplants should also be vaccinated (128).

The safety of hepatitis A vaccine during pregnancy has not been determined. However, because the vaccine is produced from inactivated HAV, the theoretical risk to the fetus is low. Nonetheless, immunization is typically not performed during pregnancy unless the risk of exposure to HAV is very high.

Vaccine immunogenicity

After a primary vaccination course, most studies have shown a seroconversion rate approaching 100% in both children and adults (177). Inactivated HAV vaccine produces substantially higher titers of circulating anti-HAV antibody than administration of protective doses of IG. Since long-term persistence of antibody has been demonstrated, HAV booster vaccination is not necessary after the primary series.

Two main groups have shown a diminished response to HAV vaccine: patients with advanced liver disease and those who are immunocompromised. In one study, rates of seroconversion were lower in those with decompensated cirrhosis (66%) compared with those with compensated cirrhosis (98%) (178). Patients with low CD4 counts (< 300 cells/mm^3) also experience lower seroconversion rates; persons with advanced human immunodeficiency virus (HIV) infection show seroconversion rates ranging from 52% to 94% (179). Patients who are receiving immunosuppressive medications respond well to a two-dose vaccination series but have suboptimal response to one dose (180).

Diminished vaccine response has been observed in infants with passively acquired antibody as the result of previous maternal HAV infection (128), which is why hepatitis A vaccination is deferred until the infant is 1 year old. In the majority of studies, all infants subsequently had protective levels of antibody, but the final levels were substantially lower than those of infants born to anti-HAV-negative mothers and vaccinated according to the same schedule. Despite lower antibody levels after the primary series, the majority of infants with passively acquired antibody respond to a booster dose 1 to 6 years later (128). Passively acquired antibody declines to undetectable levels in the majority of infants by 1 year of age. After that time, hepatitis A vaccine is highly immunogenic, regardless of maternal anti-HAV status.

In populations with high rates of previous HAV infection, prevaccination testing may be considered to reduce costs by not vaccinating persons who are already immune. However, prior infection does not pose any special risk for vaccination. Postvaccination testing is not indicated because of the high rate of vaccine response. Furthermore, not all testing methods approved for routine diagnostic use in the United States have the sensitivity to detect low (but protective) anti-HAV titers that may be present after vaccination.

Side Effects and Adverse Events

An estimated 1.3 million persons in Europe and Asia were vaccinated with Havrix before its licensure in 1995 and no serious adverse events were reported (128). Since the institution of routine childhood vaccination in the United States, no serious adverse events have been reported. Among adults, the most frequently reported adverse events occurring less than 3 days after administration were soreness at the injection site (56%), headache (14%), and malaise (7%) (181). Vaccination of a person who is immune because of previous infection does not increase the risk for adverse events.

Immunization Policies and Vaccine Coverage

Vaccination policies range from being part of a national universal immunization program for children to targeting the vaccine exclusively to high-risk groups. Immunization programs have been very successful in most cases and have reduced the incidence of infection to up to 90%. Countries that have implemented a universal immunization program for children include Argentina, Israel, Italy, Spain, and the United States (118). Targeted policies for travelers have also been effective.

Vaccine coverage in countries that have implemented universal immunization programs has varied. In Argentina, approximately 95% of children were vaccinated in 2006 (182), but in the United States, coverage has ranged from 49.7% to 57.5% between 2010 and 2014 (134). Vaccination coverage in the United States has been slowly increasing each year by approximately 2% since 2010, but there is still room for improvement. In contrast to vaccination coverage rates among children, the rate among adults in the United States is substantially lower (132). In 2013, estimated hepatitis A vaccination coverage was only 12.3% among adults aged 19 to 49 years and 5.4% among adults older than 50 years. Coverage was higher among Asians (16.1%) compared with whites (12.6%) and Hispanics (10.6%). Surprisingly, only 13.3% of adults aged more than 19 years with chronic liver disease were vaccinated.

Pre- Versus Postexposure Prophylaxis

Postexposure Prophylaxis of Hepatitis A

Active immunization with vaccine and passive immunization with IG have equivalent efficacy when used for

TABLE 3. Summary of recommendations for prevention of hepatitis A after exposure to hepatitis A virus (HAV) and in departing international travelers

	Hepatitis A vaccine	Immune globulin
Postexposure prophylaxis	1) Healthy persons aged 12 mo to 40 yr at the age-appropriate dose 2) Healthy persons aged > 40 yr if IG cannot be obtained	1) Persons aged >40 yr 2) Children aged <12 mo 3) Immunocompromised persons and persons with chronic liver disease, or for whom vaccine is contraindicated
International travel[a]	For most healthy persons, one dose of single antigen before travel	Persons who elect not to receive vaccine, are aged <12 mo, or are allergic to a vaccine component
	Older adults, immunocompromised persons, and persons with chronic liver disease or other chronic medical conditions planning to depart to an area in ≤2 weeks should receive the initial dose of vaccine and also simultaneously can be administered IG at a separate anatomic site	

[a]To countries with high or intermediate endemicity for hepatitis A
IG=immune globulin.

postexposure prophylaxis (172). Among 1,090 healthy individuals (aged 2 to 40 years) randomly assigned to vaccine or IG within 14 days of exposure, symptomatic HAV infection occurred in a similar proportion of patients in both groups (4.4% versus 3.3%, respectively) (172). Following this study, U.S. guidelines were revised to allow for hepatitis A vaccine to be used after exposure to prevent infection in healthy persons aged 1 to 40 years. Such persons should receive a single dose of hepatitis A vaccine or IG (0.02 ml/kg) as soon as possible, but ideally no later than 2 weeks after exposure (172) (Table 3). Vaccine is preferred over IG in this setting due to long-term protection and ease of administration. However, in children younger than 12 months, or individuals aged more than 40 years, and in immunocompromised persons, persons with chronic liver disease, and persons for whom HAV vaccine is otherwise contraindicated, IG should be given if possible. If immunization against HAV is otherwise warranted, a dose of vaccine should be given simultaneously with IG.

Postexposure prophylaxis should be given to close personal contacts of individuals with serologically confirmed HAV who have not been vaccinated previously, both household and sexual contacts, as well as persons who have recently shared illicit drugs with someone with hepatitis A. Postexposure prophylaxis is also warranted for unvaccinated staff and attendees of child care centers or homes if one or more cases of hepatitis A is recognized in children or employees, or if cases are recognized in two or more households of center attendees. When an outbreak occurs in a day care center, prophylaxis should be considered for members of households that have diapered children attending the center. If a food handler is diagnosed with hepatitis A, then prophylaxis should be given to other food handlers at the same location. However, prophylaxis is not indicated when only a single hepatitis A case has been identified in a school, hospital, or office setting.

Prevention of Hepatitis A in International Travelers

In June 2007, the ACIP concluded that hepatitis A vaccine alone provides protection for healthy international travelers aged more than 40 years (Table 3). The first dose of vaccine should be given as soon as travel is considered. However, for optimal immediate protection, IG can be considered in addition to vaccine and should be given to older adults, immunocompromised persons, and individuals with chronic liver disease who are traveling to an endemic area within 2 weeks. Travelers who are allergic to a vaccine component or who are aged less than 12 months should receive a single dose of IG, which provides protection for up to 3 months (Table 3) (128).

TREATMENT

Treatment of typical hepatitis A is supportive; there are no approved antiviral agents that are effective against HAV. Avoiding hepatotoxic medications and abstaining from alcohol is recommended. Hospitalization is rarely needed but occasionally age-appropriate management of nausea and diarrhea, including intravenous hydration, is required. Bed rest has no specific benefit. In contrast to typical acute hepatitis A, in which corticosteroids should never be used, the duration and degree of symptoms associated with cholestatic hepatitis may be reduced by a short course of corticosteroids (149).

Many agents have been studied for the treatment of fulminant hepatitis (e.g., corticosteroids, prostaglandin E, IFN, and ribavirin) with inconclusive results. Liver transplantation is the only potentially successful intervention, although criteria for selecting patients for transplantation have been difficult to establish. Survival is moderately high, even for patients with coma (approximately 66%) and no single factor is predictive of a poor outcome. Overall survival is 55% to 75% among patients undergoing transplantation (153). There is no conclusive evidence that HAV is able to reinfect the transplanted liver.

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Human Caliciviruses

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Norwalk virus (NV) was first recognized from an outbreak of epidemic gastroenteritis in an elementary school in Norwalk, Ohio in 1968, in which 50% of the students and teachers became ill and secondary cases occurred in 32% of family contacts (1). Subsequently, NV was visualized using immune electron microscopy (IEM) and described as a 27-nm filterable agent (2). This provided definitive proof that viruses cause diarrhea, an idea initially proposed during the 1940s and 1950s when a filterable infectious agent (although not propagated in cell culture) was passaged serially in volunteers. The first clear description of the basic virological, clinical, and immunological responses to nonbacterial infections came from studies in volunteers administered a bacteria-free fecal filtrate of NV (3). The history of these early investigations leading to visualization of the agent by IEM provides an excellent example of how major scientific advances often require and parallel new technological opportunities (4). The subsequent application of IEM to other diarrheal stool samples ultimately led to the discovery of other viral agents of gastroenteritis and hepatitis A virus (see Chapters 4, 25, 34, and 48). The later cloning and expression of the NV genome resulted in the development of new assays and reagents that permit large-scale epidemiologic studies.

VIROLOGY

Classification

Although the first report of the visualization of NV was in 1972, the virus was not classified until 1990. Classification required the successful cloning of the viral genome because NV and related agents were noncultivable in cell culture. Molecular cloning and characterization of the NV genome allowed this virus to be classified as a member of the family *Caliciviridae* (5). Caliciviruses are nonenveloped, icosahedral particles containing a single-stranded RNA of positive polarity, approximately 7.7 kilobases (kb). The name calicivirus, from the Latin *calyx* meaning "cup" or "goblet," describes the cup-shaped depressions observed by EM (see Fig. 1). Although structural studies confirm that NV contains cup-shaped depressions, these depressions are often clearer in other strains of animal and human caliciviruses (Fig. 1).

There are five genera currently recognized within the *Caliciviridae* family (6). Two of these genera (*Norovirus* [NoVs] and *Sapovirus* [SaVs]) contain human and a few animal strains while the other three genera, *Vesivirus* (e.g., vesicular exanthem virus of swine, feline calicivirus, San Miguel sea lion virus), *Lagovirus* (e.g., rabbit hemorrhagic disease virus, European brown hare syndrome virus), and *Nebovirus* (e.g., Newbury-1 virus), contain only animal strains. Classification of NV as a calicivirus based on its genomic characteristics replaced a previous interim classification in which these and other small-sized fecal viruses were classified morphologically according to whether they contained visible structural features. In this morphological system, two groups of human viruses that contain members of the *Caliciviridae* family were recognized: the classical (or morphologically typical) caliciviruses and the small round-structured viruses (SRSVs). NV was the prototype SRSV strain. Although most morphologically typical caliciviruses have been found to be SaVs and most SRSVs have been identified as NoVs, a few morphologically typical caliciviruses have been found to be NoVs. Thus, correct classification requires obtaining sequence data. Although electron microscopists in diagnostic laboratories may continue to use the morphological appearance as a tentative classification system, other rapid diagnostic assays to detect human caliciviruses (HuCVs) are available (see below).

Genogroups and Genotypes

The availability of the first NoV nucleotide sequence opened a new era in the characterization of HuCVs, including the agents previously characterized as SRSVs. Primers were designed to amplify viral sequences from clinical samples, and numerous different strains were identified. Eventually, the complete sequences of several HuCV strains were reported, and phylogenetic analyses of these and other virus strains allowed the classification of a virus strain into the genus *Norovirus* or *Sapovirus* (4).

Strains within a genus can be further subdivided based upon phylogenetic analysis of the polymerase region, the capsid region, or the third open reading frame (ORF3). Such analyses have allowed the subdivision of NoVs into seven genogroups (7) and SaVs into five genogroups (8), and virus strains in both genera into different genotypes (Tables 1 and 2). Each genogroup has been proposed to represent a separate species (9, 10), although this designation has not yet been accepted by the International Committee on Taxonomy of Viruses. Criteria for the separation of NoVs

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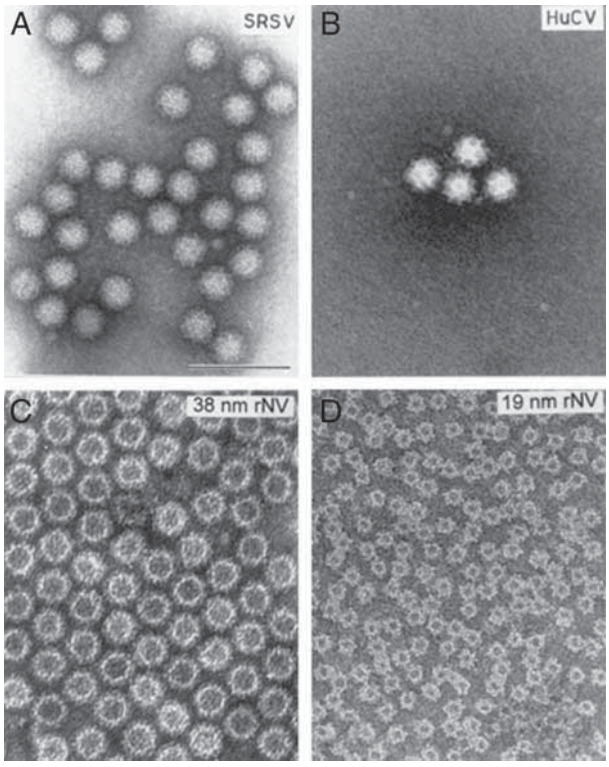

FIGURE 1 Electron micrographs of caliciviruses. Negative-stain electron micrographs of (A) an NoV (previously called small round-structured virus, or SRSV) from the stool of a volunteer given NoV/NV/8fIIa; (B) an SaV with the classical calicivirus morphologic features including distinct cuplike indentations in the surface of the particles, taken from the stool of a child and containing SaV/Sapporo; (C) 38 nm rNV particles produced and purified from insect cells infected with a baculovirus recombinant that expresses the NV ORF2; and (D) 19-nm particles produced and purified from insect cells infected with a baculovirus recombinant that expresses NV ORF2. Bar, 50 nm. Panel D adapted from White et al. (33) and courtesy of Dr. L. White.

into genogroups and further into genotypes have been developed and build upon previously reported genotypes (9). The primary designation is based upon phylogenetic analysis of the complete amino acid sequence of the major structural protein, VP1 (10). New genotypes are assigned by an international group of experts and require at least two complete capsid sequences from strains in the new genotype and differ from the closest genotype cluster by at least two standard deviations. For GI strains, this represents at least a 20% to 21% uncorrected pairwise difference of the VP1 amino acid sequence for strains in different genotypes, while for GII strains the uncorrected pairwise difference between strains in different genotypes is at least 13% to 15% (9). Sapoviruses are also classified based upon analysis of the complete VP1 sequence, although they have less genetic diversity (11).

Recombination occurs among both NoVs and SaVs, most commonly near the start of the VP1 gene, and is likely a common mechanism by which new strains are generated (12, 13). Thus, although similar phylogenetic relationships are frequently identified when both the polymerase and capsid regions of the genome are analyzed (14), discordant results can occur following a recombination event (12). This has led to the classification of norovirus strains by polymerase genotype in addition to the capsid genotype. The polymerase genotype is preceded by a capital 'P' to distinguish it from the capsid genotype. An Arabic number is used when the polymerase genotype has previously been associated with an established reference capsid genotype; a letter is used for orphan polymerase sequences. To date, P types have only been assigned to GI and GII strains (Table 1). At least 1300 nucleotides (nt) of sequence at the 3' end of the first open reading frame are required to assign a new polymerase genotype.

The formal designation of norovirus strains has been proposed to include the genogroup, the host (e.g., human, bovine, porcine), a two-digit country of origin, the genogroup and genotype (and variant name, if appropriate), and the strain name. Thus, the original Norwalk virus is designated as GI/Hu/US/1968/GI.P1-GI.1/Norwalk while a GII.4 variant is GII/Hu/GB/2010/GII.P4-New Orleans2009/London48 (9).

Serotypes and antigenicity

Norovirus and sapovirus serotypes are not yet defined due to the lack of robust cultivation systems. However, based on cross-challenge studies in volunteers and some comparisons of different prototype particles by immune electron microscopy (IEM) and enzyme-linked immunosorbent assay (ELISA), an initial proposal (before classification into genera) identified at least five serotypes, represented by NV, Hawaii virus (HV), Snow Mountain virus (SMA), the Taunton virus, and Sapporo virus (SV) (15). Subsequently, additional antigenic groups were proposed based on IEM studies. These serotype designations assume that antibody reactivity by IEM reflects reactivity of antibody with neutralization epitopes on the surface of particles. However, some polyclonal and monoclonal antibodies that bind virus do not block virus binding to cells (16). The antigenic relationships between a subset of these viruses have been evaluated in ELISAs using hyperimmune antisera generated against recombinant virus-like particles (VLPs). Although only limited comparisons have been performed to date, viruses belonging to distinct genotypes are antigenically distinct from strains belonging to other genotypes for both NoVs (17) and SaVs (18). Better characterization of distinct serotypes will become possible as cell culture methods are developed.

Recombinant VLPs have been used to characterize serological immune responses of individuals involved in experimental human infection studies (19, 20) and in outbreaks of NoV infection (21). The likelihood of detecting a serological response is greater when the antigen used is derived from a virus in the same genotype, but there are not enough data available at this time to determine the significance (in terms of biologic or type differences) of genotype designations. For example, patients infected with GI.1 viruses can show cross-reactive immune responses to other genogroup I viruses, although usually at a lower frequency and magnitude compared to the response to the homologous strain (19, 20). In some instances, persons infected with genogroup I viruses from genotypes distinct from NV (GI.1) show seroresponses to NV VLPs that are similar to those seen when the infecting strain is from the NV genotype (21). In contrast, patients infected with genogroup II viruses have had such "homologous" responses to Toronto virus (GII.3) and Hawaii virus (GII.1) VLPs only when the infecting strain is in the genotype from which the test antigen strain was derived (21). Cross-genogroup responses have also been observed, and it is likely that a person's past NoV infection history influences the occurrence of such responses.

TABLE 1 Prototype strains *Norovirus* strains by genogroups and genotypes based upon (9, 10, 7)

Capsid genogroup genotype	Capsid prototype strain	GenBank number	Polymerase genogroup genotype*	Polymerase prototype strain	GenBank number
I.1	GI/Hu/US/1968/GI.1/Norwalk	M87661	I.P1	GI/Hu/US/1968/GI.P1/Norwalk	M87661
I.2	GI/Hu/UK/1991/GI.2/Southampton	L07418	I.P2	GI/Hu/UK/1991/GI.P2/Southampton	L07418
I.3	GI/Hu/SA/1990/GI.3/Desert Shield 395	U04469	I.P3	GI/Hu/US/1998/GI.P3/VA98115	AY038598
I.4	GI/Hu/JP/1987/GI.4/Chiba 407	AB022679	I.P4	GI/Hu/JP/1987/GI.4/Chiba 407	AB042808
I.5	GI/Hu/UK/1989/GI.5/Musgrove	AJ277614	I.P5	GI/Hu/SE/2005/GI.P5/07_1	EU007765
I.6	GI/Hu/DE/1997/GI.6/BS5(Hesse3)	AF093797	I.P6	GI/Hu/DE/1997/GI.P6/BS5(Hesse3)	AF093797
I.7	GI/Hu/UK/1994/GI.7/Winchester	AJ277609	I.P7	GI/Hu/SE/2008/GI.P7/Lilla Edet	JN603251
I.8	GI/Hu/US/2001/GI.8/Boxer	AF538679	I.P8	GI/Hu/US/2008/GI.P8/890321	GU299761
I.9	GI/Hu/CA/2004/GI.9/Vancouver730	HQ637267	I.P9	GI/Hu/FR/2004/GI.P9/Chatellerault709	EF529737
			I.Pa	GI/Hu/SA/1990/GI.Pa/Desert Shield 395	U04469
			I.Pb	GI/Hu/JP/2002/GI.Pb/WUG1	AB081723
			I.Pc	GI/Hu/JP/2000/GI.Pc/SzUG1	AB039774
			I.Pd	GI/Hu/FR/2003/GI.Pd/Vesoul576	EF529738
			I.Pf	GI/Hu/JP/1979/GI.Pf/Otofuke	AB187514
II.1	GII/Hu/US/1971/GII.1/Hawaii	U07611	II.P1	GII/Hu/US/1971/GII.P1/Hawaii	U07611
II.2	GII/Hu/UK/1994/GII.2/Melksham	X81879	II.P2	GII/Hu/UK/1994/GII.P2/Melksham	X81879
II.3	GII/Hu/CA/1991/GII.3/Toronto 24	U02030	II.P3	GII/Hu/CA/1991/GII.P3/Toronto 24	U02030
II.4	GII/Hu/UK/1993/GII.4/Bristol	X76716	II.P4	GII/Hu/UK/1993/GII.P4/Bristol	X76716
II.5	GII/Hu/UK/1990/GII.5/Hillingdon	AJ277607	II.P5	GII/Hu/HU/1999/GII.P5/MOH	AF397156
II.6	GII/Hu/UK/1990/GII.6/Seacroft	AJ277620	II.P6	GII/Hu/JP/2002/GII.P6/Saitama U16	AB039778
II.7	GII/Hu/UK/1990/GII.7/Leeds	AJ277608	II.P7	GII/Hu/JP/2002/GII.P7/Saitama U4	AB039777
II.8	GII/Hu/NL/1998/GII.8/Amsterdam	AF195848	II.P8	GII/Hu/JP/2002/GII.P8/Saitama U25	AB039780
II.9	GII/Hu/US/1997/GII.9/VA97207	AY038599			
II.10	GII/Hu/DE/2000/GII.10/Erfurt 546	AF427118			
II.11	GII/Sw/JP/1997/GII.11/Sw918	AB074893	II.P11	GII/Sw/JP/1997/GII.P11/Sw918	AB074893
II.12	GII/Hu/UK/1990/GII.12/Wortley	AJ277618	II.P12	GII/Hu/JP/2005/GII.P12/Sakai/04-179	AB220922
II.13	GII/Hu/US/1998/GII.13/Fayetteville	AY113106	II.P13	GII/Hu/FR/2004/GII.P13/Briancon870	EF529741
II.14	GII/Hu/US/1999/GII.14/M7	AY130761			
II.15	GII/Hu/US/1999/GII.15/GII.15/J23	AY130762	II.P15	GII/Hu/JP/2006/GII.P15/Hiroshima66	AB360387
II.16	GII/Hu/US/1999/GII.16/Tiffin	AY502010	II.P16	GII/Hu/DE/2000/GII.P16/Neustrelitz260	
II.17	GII/Hu/US/2002/GII.17/CS-E1	AY502009			
II.18	GII/Sw/US/2003/GII.18/OH-QW101	AY823304	II.P18	GII/Sw/US/2003/GII.P18/OH-QW101	AY823304
II.19	GII/Sw/US/2003/GII.19/OH-QW170	AY823306			
II.20	GII/Hu/DE/2002GII.20/Luckenwalde591	EU373815	II.P20	GII/Hu/GE/2005/GII.P20/Leverkusen267	EU424333

(*Continued on next page*)

TABLE 1 Prototype strains *Norovirus* strains by genogroups and genotypes based upon (9, 10, 7) (*Continued*)

Capsid genogroup genotype	Capsid prototype strain	GenBank number	Polymerase genogroup genotype*	Polymerase prototype strain	GenBank number
II.21	GII/Hu/IQ/2003/GII.21/IF1998	AY675554	II.P21	GII/Hu/FR/2004/GII.P21/Pont de Roide673	AY682549
II.22	GII/Hu/JP/2002/GII.22/Yuri	AB083780	II.P22	GII/Hu/JP/2003/GII.P22/Hokkaido133	AB212306
II.23	GII/Hu/JP/2004/GII.23/Chiba040502	AJ844470			
			II.Pa	GII/Hu/JP/2004/GII.Pa/SN2000JA	AB190457
			II.Pc	GII/Hu/US/1976/GII.Pc/Snow Mountain	AY134748
			II.Pe	GII/Hu/JP/2007/GII.Pe/OC07138	AB434770
			II.Pf	GII/Hu/FR/1999/GII.Pf/S63	AY682550
			II.Pg	GII/Hu/AU/1983/GII.Pg/Goulburn Valley	DQ379714
			II.Ph	GII/Hu/JP/1997/GII.Ph/OC97007	AB089882
			II.Pj	GII/Hu/GR/1997/GII.Pj/E3	AY682552
			II.Pk	GII/Hu/JP/1996/GII.Pk/OC96065	AF315813
			II.Pm	GII/Hu/IN/2006/GII.Pm/PunePC24	EU921353
			II.Pn	GII/Hu/CN/2007/GII.Pn/Beijing53931	GQ856469
			II.Pp	GII/Hu/JP/2011/GII.Pp/Yuzawa Gira2HS	KJ196291
III.1	GIII/Bo/DE/1980/GIII.1/Jena	AJ011099			
III.2	GIII/Bo/NL/1998/GIII.2/CH126	AF097917			
III.3	GIII/Ov/NZ/2007/GIII.3/Norsewood30	EU193658			
IV.1	Hu/NoV/Alphatron/1998/NL	AF195847			
IV.2	Lion/NoV/387/2006/IT	EF750827			
V.1	Mu/NoV/MNV-1/2003/US	AY228235			
V.2	GV/Rn/HK/2011/GV.2/HKU_CT2	JX486101			
VI.1	GVI/Ca/IT/2007/GVI.1/Bari 91	FJ875027			
VI.2	GVI/Ca/PT/2007/GVI.2/Viseu C33	GQ443611			

*Polymerase genotypes designated by a letter are ophan genotypes that have not been previously associated with an established reference capsid genotype.

TABLE 2 Prototype *Sapovirus* strains by capsid genogroups and genotypes (8)

Genogroup genotype	Prototype strain	GenBank number
I.1	GI/Hu/JP/1982/GI.1/Sapporo	U65427
I.2	GI/Hu/US/1994/GI.2/Parkville	U73124
I.3	GI/Hu/SE/1997/GI.3/Stockholm 318	AF194182
I.4	GI/Hu/JP/2000/GI.4/Chiba000496	AJ606693
I.5	GI/Hu/JP/2000/GI.5/Ehime643	DQ366345
I.6	GI/Hu/JP/2000/GI.6/Chiba 000764	AJ606694
I.7	GI/Hu/JP/2004/GI.7/Ehime 04-1680	AB258428
II.1	GII/Hu/UK/1992/GII.1/London	U95645
II.2	GII/Hu/MX/1990/GII.2/Mex340	AF435812
II.3	GII/Hu/JP/2004/GII.3/C12	AY603425
II.4	GII/Hu/JP/2003/GII.4/Kumamoto6	AB429084
II.5	GII/Hu/US/2000/GII.5/Cruise ship	AY289804
II.6	GII/Hu/TH/2004/GII.6/SaKaeo-15	AY646855
II.7	GII/Hu/JP/2008/GII.7/20072248	AB630067
III.1	GIII/Sw/US/1980/GIII.1/PEC-Cowden	AF182760
IV.1	GIV/Hu/US/1990/GIV.1/Hou7-1181	AF435814
V.1	GV/Hu/AR/1995/GV.1/Arg39	AY289803
V.2	GV/Hu/JP/2012/GV.2/NGY-1	AB775659
V.3	GV/Sw/JP/2008/GV.3/TYMPo239	AB521771
V.4	GV/Sea lion/US/2011/9775	JN420370

Viruses with similar antigenic reactivity have been identified at different times and geographic locations such that viruses antigenically similar to some prototype viruses are still circulating, and these strains show relatively good conservation of amino acid sequences over long periods of time (22). On the other hand, viruses belonging to some genotypes appear to undergo antigenic drift through epochal evolution. This phenomenon is best described for GII.4 viruses, with new variants emerging every 2 to 4 years over the past two decades. Analysis of the amino acid sequences of the major capsid protein of GII.4 strains found that changes preferentially accumulate in the outer portion of the capsid protein (the P domain, see below) (23). The emergence of new variants with the concomitant occurrence of epidemic disease suggests that immunity in the human population drives antigenic drift and the evolution of at least some norovirus genotypes (24).

COMPOSITION

Virion Morphology, Structure, Size

NV originally was described as a 27-nm particle based on analysis of particles obtained from stools that were aggregated with antibody (2). Structural analysis of particles from stool is limited by the necessity to perform IEM due to the low numbers of particles present in most samples. Based on such micrographs, NV appeared to have a feathery outer edge that lacked a definitive surface substructure (Figure 1a); in certain orientations, NV has minor surface indentations.

A more precise description of the structure of NV is now available based on the analysis of recombinant Norwalk virus (rNV) particles produced in insect cells infected with a baculovirus recombinant expressing the cDNA that encodes the capsid proteins (Fig. 1c and Fig. 2) (25). The NV capsid

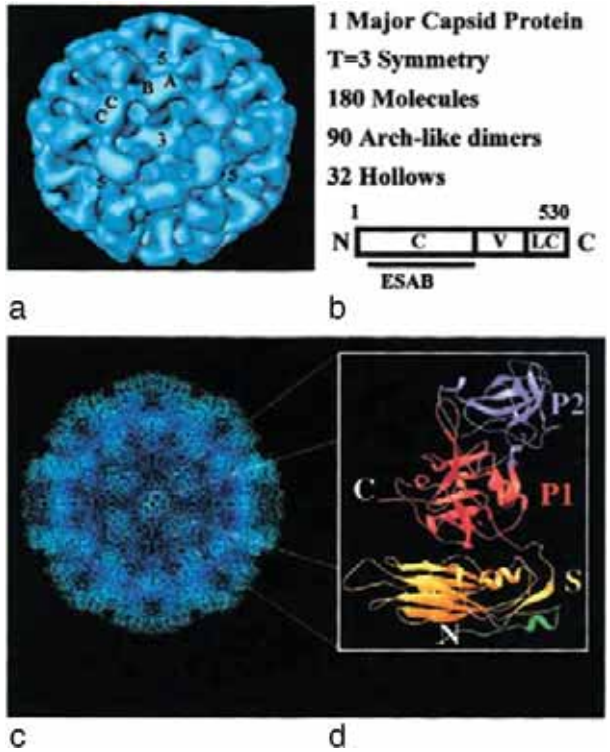

FIGURE 2 Structure of Norwalk virus particles. (a) Surface representation of the three-dimensional surface structure of rNV particles viewed along the icosahedral 3-fold axis. This structure was determined by image processing of the rNV particles shown in Figure 1c. The rNV particles have a distinct architecture and they exhibit T = 3 icosahedral symmetry; the 2-fold, 3-fold, and 5-fold axes of symmetry are shown. Cuplike depressions are evident at the 3-fold and 5-fold axes. The capsid structure is made up of 90 archlike dimers of a single protein that form two types of capsomers; A/B capsomeres (n=60) surround the 3- and 5-fold axes of symmetry and C/C capsomers (n=30) are located at the 2-fold axes of symmetry. (b) Summary of the properties of the single protein that makes up the NV capsid structure. A linear schematic of the three domains (c, conserved; v, variable; lc, less conserved) in the single capsid protein and a region predicted to fold into an eight-stranded antiparallel beta-barrel is shown. (c) X-ray crystallographic structure of rNV capsid at 3.4 Å resolution, as viewed along icosahedral 2-fold axis. Only backbone atoms of the 180 subunits are depicted, and the structure is depth-cued, with deeper blue at lower radii and lighter blue at higher radii. (d) Ribbon presentation of the C subunit of rNV capsid protein. N-terminal arm, S domain, P1 and P2 subdomains are colored in green, yellow, red, and blue, respectively. N- and C-termini of the capsid protein are indicated. The C-terminus faces a hollow, the N-terminus faces the interior of the capsid, and the P2 subdomain faces the exterior of the capsid. Figures a, c, and d kindly provided by B.V.V. Prasad; modified from Prasad et al. (27, 28), with permission.

is composed of 180 copies of a major polypeptide (VP1) that folds to produce the capsid structure and a few (<5) copies of another polypeptide (VP2) (26). By negative-stain electron microscopy, rNV particles have a similar morphology to the native NV (Fig. 1). The rNV particles have a distinct architecture and exhibit T=3 icosahedral symmetry (Fig. 2) (27). The major capsid protein folds into 90 dimers that form a shell domain from which archlike capsomers protrude (Fig. 2 b–d). The crystallographic structure of rNV particles

shows that the major capsid protein is folded into two domains, a shell domain and a protruding domain. The shell domain has a classical eight-stranded beta sandwich motif found in many viral capsid structures, and the amino acid sequence of this domain is the most conserved region of the capsid protein sequence (28). The arches are made from the protruding domain and are arranged in such a way that there are large hollows at the icosahedral 5- and 3-fold positions (Fig. 2b), which appear as cuplike structures in classical caliciviruses. The three-dimensional structures of other caliciviruses are quite similar to that of NV (29). The S domain serves as an icosahedral scaffold (Fig. 2d). The P2 domain is an insert into the P1 domain, and it has the greatest sequence and structural variability in the protein. The protruding domain of the classical calicivirus is longer than that for NV, and the shape of the top of the arch also differs in such a way that the NV would show a feathery appearance by negative stain EM.

These three-dimensional structures provide independent evidence of the similarities of distinct morphologic types of caliciviruses, as well as structural information on where antigenic epitopes and the cellular attachment site in the virus capsid might be located. Although not yet proven to be cellular receptors for NoVs, histoblood group antigens such as the type A and B trisaccharides bind by extensive hydrogen bonding to the outer portion of the P2 domain of GI and GII NoVs, although at distinct locations on the capsid (30, 31). The greatest sequence variation in the major capsid protein is in the top of the protruding arch region, a region that contains distinct epitopes that change among GII.4 variants (28, 32).

Preparations of recombinant NoV [rNV and rMexico virus [GII.3]) particles also contain smaller-sized particles (∽ 19 nm) (Fig. 1d) (33). These particles probably represent an alternative assembly of the single capsid protein, and they possess similar binding and antigenic properties compared to the larger particles. Such smaller-sized empty particles also have been observed in stools of children infected with these viruses, but it is not known if these smaller particles have any distinct biological properties.

Genome Organization and Viral-Specific Proteins

The first sequence of the NV genome was obtained from sequencing cloned DNA (cDNA) from virus partially purified from stools obtained from volunteer studies (5, 34). The full-length genomic sequence is known for more than 20 NoV strains and several SaV strains. Partial sequences of hundreds of other NoVs and SaVs have also been determined.

The genome is a positive sense, polyadenylated, single-stranded RNA approximately 7.4 to 8.3 kb in length, excluding the 3' polyadenylated tail (6). After a short noncoding region at the 5' sequence, the genome of the NoVs is predicted to encode three open reading frames (ORFs), as shown in Fig. 3. The first (ORF1) and second (ORF2) ORF sequences overlap by a short, variable (14 to 17) number of nucleotides. ORF3 is in a separate reading frame from ORF2, overlapping ORF2 by one (or a few) nucleotide(s). SaVs have only two ORFs, with the genes encoded by NoV ORF1 and ORF2 in the same ORF (ORF1) of the SaVs (Fig. 3).

The longest NoV ORF, ORF1, encodes a polyprotein precursor of nonstructural proteins based on the identification of sequences similar to the picornavirus nonstructural proteins (Table 3). *In vitro* translation of the ORF1 from Southampton virus (a GI.2 NoV) yields a polyprotein that is cotranslationally cleaved to give three major products: a 48 kDa N-terminal protein (NS1-2), a 41 kDa protein NTPase (NS3), and a 113 kDa protein that is homologous to the 3ABCD region of picornaviruses. The 113 kDa protein can be cleaved further into a 22 kDa protein (NS4), a 16 kDa protein (NS5, VPg), a 19 kDa protein (NS6, the 3C protease), and a 57 kDa protein (NS7, the RNA-dependent RNA polymerase). Similar findings have been noted when the ORF1 of a GII. NoV (NoV/Camberwell 101922/1994/AU) virus) is expressed in COS cells using a simian virus 40-based expression vector (35). The structures of a GI.1 NoV protease and of a GII.4 polymerase have been solved (36, 37), and these proteins are being evaluated as targets in the future developments of antiviral drugs (38, 39).

Proteins expressed from ORF1 are immunoreactive based on detection by human immune sera of a fusion protein expressed in a λgt11 library and immunoprecipitation of a 57 kDa protein expressed from ORF1 in insect cells (40, 34) and the development of immune responses to the NV protease by infected persons (41). These results indicate that infected individuals make antibodies to proteins other than the capsid protein, an observation that should be considered when interpreting early data on the antigenic relatedness of these viruses determined by RIA or ELISA using stool extracts as antigen and sera from adult volunteers.

The second ORF of NV encodes a protein of 530 amino acids with a calculated molecular weight of 56,571, similar in size to the viral capsid protein (Fig. 3). The NV ORF2 contains a conserved amino acid motif of PPG that also is found in the picornavirus capsid protein VP3 (34). Expression of ORF2 and ORF3 in insect cells infected with a baculovirus recombinant containing this gene, and the expression of ORF2 alone in cell-free translation systems produces products similar in size to that observed for the capsid protein of native NV particles, confirming that ORF2 encodes the capsid protein that self-assembles into VLPs (Fig. 1c).

A soluble viral antigen with an apparent molecular weight of approximately 30,000 kilodaltons (kDa) is excreted in the stools of volunteers infected with NV (34), and this antigen has been shown to result from the specific cleavage of the capsid protein (42). The amino terminus of the soluble protein detected in the stools of volunteers given NV is the same as that obtained following trypsin treatment of preparations of rNV particles (42). However, this cleavage product is not made from intact capsid protein but from soluble capsid protein; the cleavage site is buried in the hinge region between the protruding domain and shell domain within intact particles. This indicates that this specific cleavage may not be important in activation of infectivity; it remains unknown if it affects the immunogenicity or pathogenicity of these viruses.

ORF3 is at the 3' end of the genome. For NV, it encodes a small protein of 212 amino acids with a molecular weight of 22.5 kDa and a very basic charge (isoelectric point of 10.99). Sequences of the ORF3 of other NoVs indicate that the protein encoded by this gene ranges from 211 to 268 amino acids. The NV ORF3 protein is a minor structural protein, present in both native virions and VLPs expressed from a baculovirus recombinant containing both ORF2 and ORF3 (26). In native virions, the apparent molecular weight of the ORF3 protein was found to be 35 kDa in size. The higher molecular weight apparently results from phosphorylation of the ORF3 protein, based on the observation that the 35 kDa protein is lost following phosphatase treatment of baculovirus expressed ORF3 recombinants (26). The role of the ORF3 protein is still unknown, although it may be involved in nucleic acid binding and encapsidation of the viral RNA (26).

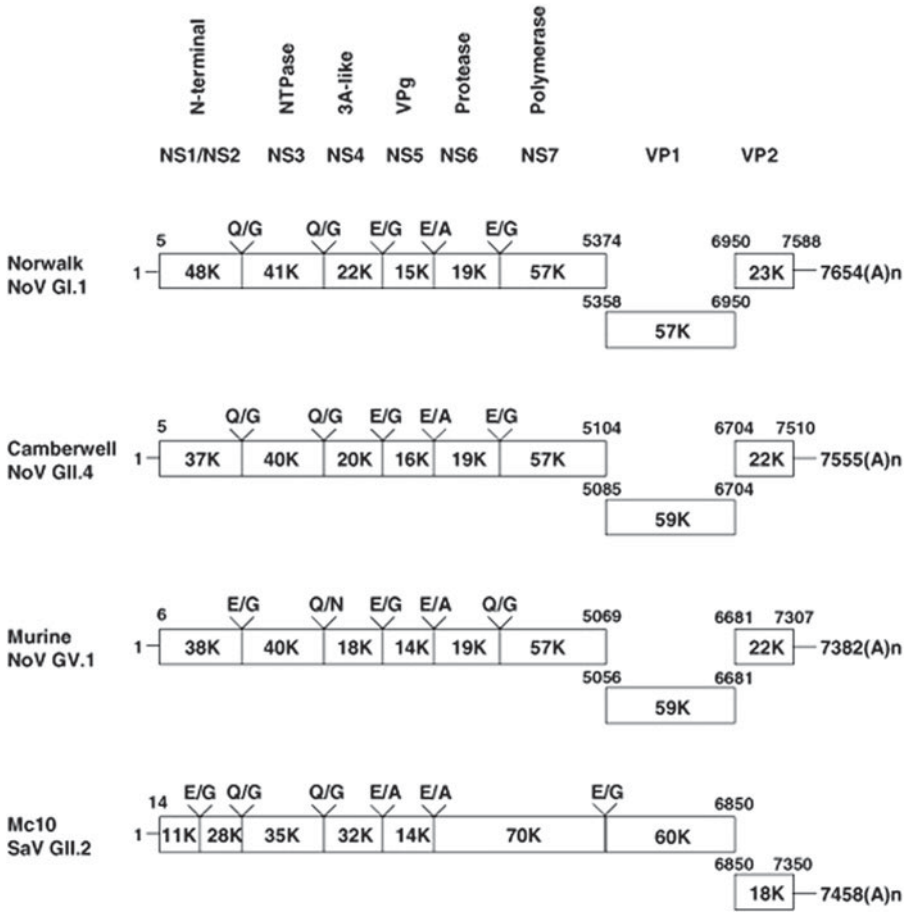

FIGURE 3 Genomic organization of noroviruses and sapoviruses. Schematic of the genomic organization of two human genogroup I and II NoVs, the murine genogroup V NoV and a human genogroup II SaV. The NoVs have three predicted ORFs that include (1) ORF1, a polyprotein that contains the nonstructural proteins NS1-NS7; (2) ORF2, the major capsid protein (VP1); and (3) ORF3, a minor capsid protein (VP2). For SaVs, ORF1 is longer and contains the major capsid protein, VP1 (see text for details). Nucleotide numbers denoting ORFs are indicated for each of the viruses. Molecular weights of each of the viral proteins are also indicated. Among the NoVs, NS1/NS2 forms a single protein, and for the SaV strain indicated, NS6/NS7 forms a single protein. This information is compiled from GenBank sequences M87661 (NV), AF145896 (Camberwell), AY228235 (Murine), and AY237420 (Mc10) and selected references (35, 43, 44, 160, 161).

TABLE 3 Human calicivirus structural and nonstructural proteins

Name of protein	Protein size in kDa	ORF	Function	Comments
N-terminal (NS1/NS2)	37–41	ORF1	Unknown	NS1/NS2 is cleaved into two peptides in some NoV and SaV strains
NTPase (NS3)	40–41	ORF1	NTPase	Binds and hydrolyzes NTP
3A-like (NS4)	19–32	ORF1	Unknown	Location in ORF1 analogous to picornavirus 3A protein
VPg (NS5)	14–16	ORF1	VPg	Binds cellular initiation factors involved in protein synthesis
Protease (NS6)	13–19	ORF1	Cysteine protease	Mediates cleavage of ORF1-encoded polyprotein
Polymerase (NS7)	57	ORF1	RNA-dependent, RNA polymerase	May also have protease and polymerase function as uncleaved precursor protein (NS6/NS7)
VP1	58–60	ORF2 (NoV) ORF1 (SaV)	Major structural protein	180 copies per virion, part of ORF1 in SaVs
VP2	12–29	ORF3 (NoV) ORF2 (SaV)	Minor structural protein	A few copies per virion of this basic protein

The overall genomic organization of SaVs differs from that of NoVs, more closely resembling that of the animal calicivirus RHDV. As noted earlier, the VP1 gene lies at the 3'end of ORF1. *In vitro* translation experiments show the ORF1 of SaV/Mc10/2000/Thailand is cleaved into the same proteins as were described for NoVs, with the exception that the N-terminal protein is cleaved into two peptides, NS1 (11 kDa) and NS2 (28 kDa), and no cleavage of the protease and polymerase (NS6-7) occurs (43, 44). ORF2 is in frame −1 relative to ORF1 at the 3' end of the genome and it could encode a basic, hydrophilic protein that contains no cysteine residues and is analogous to ORF3 of the NoVs.

BIOLOGY

Norovirus Replication Strategy

Many human NoVs bind to carbohydrate histoblood group antigens (HBGAs) present on the surface of epithelial cells (45). There are strain-specific differences in NoV recognition of HBGAs, and for some NoVs, no HBGA to which the virus binds has been identified (46). The specific HBGA to which a NoV strain binds has been proposed to be a potential viral receptor based upon the lack of susceptibility to infection of persons who have genetic mutations that prevent the expression of the HBGA on the surface of the cell (47, 48, 49). For example, mutations in the fucosyltransferase 2 gene that inactivate or prevent expression of the corresponding enzyme prevent expression of secretor-type HBGAs on gut epithelium and are associated with absolute resistance to GI.1 strains and to most GII.4 strains. A number of HBGA-binding patterns exist for the different NoV strains such that most, if not all, persons have the potential to be susceptible to at least some NoV strains (46). To date, HBGAs have not been found to play a role in the binding of human SaVs to epithelial cells (13).

The mechanism by which the human NoV strains enter the cell is not known. GII.4 VLPs induce invaginations after binding to glycosphingolipids and causing their clustering on the surface of giant unilamellar vesicles; this may be an initial step in virus cell entry (50). Studies of murine NoV and porcine enteric calicivirus (a SaV) indicate that virus entry involves trafficking through endosomes (51). After entry, the NoV genomic RNA serves as a messenger RNA template from which the nonstructural proteins are produced. A subgenomic RNA containing ORF2 and ORF3 is produced during viral replication and serves as a template for production of the structural proteins, VP1 and VP2. Besides the genomic RNA, a major species of subgenomic RNA (over 2 kb) has also been observed in stools of a volunteer infected with NV (40). The availability of cell culture for porcine enteric calicivirus and murine NoV and reverse genetics systems will allow further characterization and improved understanding of the replication strategies of these viruses (52, 53, 54).

Host Range and Animal Infection

Natural infections with HuCVs suggest that these viruses are largely species-specific. Attempts to experimentally transmit NV and other HuCVs to a wide range of animals (chickens, mice, guinea pigs, rabbits, kittens, puppies, piglets, calves, baboons, and various monkey species) have largely been unsuccessful. Some rhesus monkeys and rabbits have seroconverted when fed HuCV, and a chimpanzee fed NV shed soluble antigen and responded serologically. More recently, replication of human GII.4 NoV strains have been successfully demonstrated in gnotobiotic piglets and calves and in immunodeficient Balb/c mice inoculated intraperitoneally (55, 56). Nevertheless, most available data suggest that the host range specificity for HuCVs is narrow. Animal (porcine, bovine, and lion) strains have been noted in both the NoV and SaV genera (7, 8, 9), but it is not known whether these strains can be transmitted to humans. To date, no virus strain identified in a human has had the sequence of an animal NoV. The factors responsible for host restriction are not known at this time. Animal caliciviruses (vesiviruses and lagoviruses) have been noted to cause extraintestinal disease and to have a broad host range, being able to infect more than one species of animal. Additional human illnesses and cross-transmission between species may be discovered as the HuCVs become better characterized.

Growth in Cell Culture

The HuCVs have been refractory to cultivation in cell culture and in animal models. A report noting the propagation of NoVs in suspensions containing three-dimensional aggregates of human embryonic small intestinal cells (INT-407) has not been confirmed by other investigators (57). More recently, the propagation of a GII.4 strain in a B lymphocyte line (BJAB) has been reported to be dependent on coinoculation with bacteria expressing histoblood group antigen (58). Confirmatory studies of this finding are still needed. A variety of cell lines (including human and animal cells) bind rNV particles, suggesting that host range specificity does not occur at the level of cell binding (16). In addition, NV RNA transfected into several of the mammalian cells that bind virus is infectious, producing viral particles (59). Similar findings have been observed with a NoV reverse genetics system (53).

Inactivation by Physical and Chemical Agents

Studies of the stability of the HuCVs have been done by experimental infection of humans. Consistent with the fact that these agents are the most important cause of food and waterborne disease, NV is resistant to inactivation following treatment with chlorine concentrations usually present in drinking water, and NV is more resistant to inactivation by chlorine than poliovirus 1, human rotavirus (Wa), simian rotavirus (SA11), or f2 bacteriophage (60). NV retains infectivity for volunteers following (1) exposure to pH 2.7 for 3 hours at room temperature, (2) treatment with 20% ether at 4°C for 24 hours, or (3) after incubation at 60°C for 30 minutes (61). NoVs also have retained infectivity after freezing. However, high hydrostatic pressure treatment of NoV-inoculated oysters inactivated virus infectivity in a human infection model (62), although the palatability of the oyster was adversely affected by the treatment. NoV-contaminated surfaces can be disinfected effectively using a combination of detergent and sodium hypochlorite (63). A variety of cultivable virus surrogates have been used to assess responses to different inactivation procedures and to compare the results measured using molecular assays with those for GI and GII noroviruses (64). No single surrogate satisfactorily represents results seen with human strains, and among the noroviruses there appear to be differences in susceptibility from one strain to another.

EPIDEMIOLOGY

Epidemiological studies indicate that NoVs are among the most common causes of gastroenteritis worldwide (65, 66).

Geographic and Temporal Distribution

NoVs are the major causes of epidemic gastroenteritis in both developed and developing countries (65, 66). Infections by NoVs have been detected on all continents, and these viruses appear to have a worldwide distribution. SaVs also are causes of gastroenteritis worldwide but less frequently than NoVs (8). Epidemic viral gastroenteritis usually occurs in family or community-wide outbreaks, affecting adults, school-age children, family contacts, and young children. Epidemic viral gastroenteritis is usually mild and self-limited, distinguishing it from infantile gastroenteritis caused by rotaviruses, which is generally a severe (often life-threatening) diarrheal illness in infants and young children. Infection with both NoVs and SaVs occur year round, although a distinct increase in occurrence of disease has been noted during cold weather months for both viruses (8, 67).

Incidence and Prevalence of Infection

Specific incidence data for illness associated with NoV and SaV infections are not available in the U.S., although norovirus disease burden has been estimated (68). Investigators at the CDC calculated that 19 to 21 million norovirus illness occur annually in the United States. The illnesses lead to approximately 1.7 to 1.9 million outpatient visits, 400,000 emergency department visits, 56,000 to 71,000 hospitalizations, and 570–800 deaths per year. Children under 5 years of age have the highest rates of outpatient visits and hospitalizations while almost all of the mortality occurs in adults more than 65 years of age. With the introduction of rotavirus vaccination in young children, noroviruses are replacing rotaviruses as the viral pathogen associated with the greatest impact (medically attended illnesses and hospitalizations) (69).

The CDC estimates that the average person in the United States will have approximately five norovirus illnesses over his or her lifetime (68). In the United Kingdom, the Infectious Intestinal Disease II study estimated an annual incidence of 47 norovirus illnesses per 100 person-years (70), which is similar to the estimate for the United States. SaVs were the second most common cause of viral acute gastroenteritis in the IID2 study, which is higher than other epidemiological studies have noted (70, 71). The risk of infection and illness is highest in children under 5 years of age. In low-income countries, the high prevalence of NoV detection in asymptomatic persons (either due to prolonged postsymptomatic infection or to subclinical infection) has made it difficult to assess disease burden. In a multicountry, case-control study of moderate-to-severe diarrhea (Global Enteric Multicenter Study, GEMS) of children under 5 years of age, NoV infection was not associated with a measurable attributable risk of illness in most participating countries because of the high frequency of infection in asymptomatic children (72). On the other hand, in the multicenter Malnutrition and Enteric Disease (MAL-ED) study, GII NoVs had the highest overall attributable risk as a cause of diarrhea in the first year of life (73). NoVs also have been detected in patients with gastroenteritis in the developing world and are a common cause of traveler's diarrhea (74, 75).

NV antibody seroprevalence was examined in relatively large studies performed in the 1970s using reagents from volunteers and a radioimmunoassay (RIA) or immune adherence hemagglutination assay (IAHA) (15). These studies indicated antibody to NV is acquired gradually, beginning slowly in childhood and accelerating in adult years, so that >50% of adults possess antibody to NV by age 50. Similar observations have been made in the UK, Japan, and Sweden using rNV capsid antigens, although a greater percentage of adults (89% to 98%) possessed antibody to NV. The higher detection rate for antibody likely reflects the greater sensitivity of the rNV antibody test (76). Other recombinant NoV and SaV capsid antigens have been used in seroprevalence studies in both developed and developing countries (15, 77). NoV-specific antibody is transferred transplacentally, with up to 90% or more of newborns having measurable serum antibody (77). Antibody seroprevalence declines during the first 6 months of life, and then it rises progressively thereafter as infants and young children acquire natural infection. Infection with NoVs can occur at a younger age (less than 1 year) than previously recognized.

A number of different NoV strains circulate in a community at any given time. During the epidemic period, strain diversity is greatest at the beginning of the season and declines as the season progresses (78). GI NoVs have been identified more commonly as causes of traveler's diarrhea and are also more commonly linked with shellfish-associated disease, while GII strains are most commonly associated with sporadic infection and outbreaks of gastroenteritis (66, 74, 75, 79). The reasons for the observed differences between genogroups is not known, although NV (a GI NoV) has been shown to specifically bind to oyster tissues (80, 81). GI and GII NoVs may exhibit different stabilities under different environmental conditions.

Outbreaks of Gastroenteritis

Much of our understanding of the epidemiology of NoVs has come from studying the cause of outbreaks of water- and food-borne gastroenteritis. NoVs are now recognized as the most common cause of outbreaks of nonbacterial gastroenteritis in the United States, Europe, and Japan, with 60% to >90% of these outbreaks being associated with NoV infection (66). Outbreaks have occurred in recreational camps, cruise ships, communities, hospitals, schools (elementary or college), the military, nursing homes, and families. They have been associated with contaminated drinking water, swimming water, consumption of uncooked or poorly cooked shellfish, ice, bakery products (frosting), various types of salads (potato, fruit, tossed), and cold foods (celery, melon, vermicelli, consommé, sandwiches, and cold cooked ham) (82). Outbreaks can occur year-round and affect primarily school-aged children and adults.

Infections with SaVs were first detected among young children with gastroenteritis (83), although infection of adults and the elderly also occur. They have been associated with outbreaks in orphanages, day care centers, schools, and hospital wards (13). SaVs also have been associated with food-borne outbreaks but much less frequently than NoVs (8).

Nosocomial infection with HuCVs may be quite common (84). As noted above, asymptomatic infections can occur (85, 86), and asymptomatic virus shedders may be the source of some outbreaks. NoV infections occur in immunocompromised hosts, and prolonged (>1 year) symptomatic shedding leading to severe malnutrition and dehydration has been described (87). Hospitals and long-term care facilities are among the most common settings where outbreaks occur (84). Outbreaks in hospitals in the United Kingdom have led to the closure of many hospital wards, with a high economic impact, while more than 50% of NoV outbreaks reported in the United States occur in long-term care facilities (88, 89). While NoVs have been found in the stools of HIV-positive patients, its role in the

etiology of gastroenteritis or infection in this patient population is not greater than in non-HIV-infected controls (90, 91). Outbreaks have been associated with military personnel during field or shipboard maneuvers and with the elderly, often in nursing homes or hospital settings (92, 93, 94). These infections can be devastating because of high secondary attack rates leading to sustained chains of transmission, and such outbreaks can last several months. Serial outbreaks on cruise ships have also occurred (95). Reinfections with the same agent can occur, as clearly demonstrated by the susceptibility of volunteers to symptomatic or asymptomatic infection following multiple challenges with the same infectious virus (96, 97).

Since the mid-1990s, the GII.4 genotype has been the predominant cause of NoV outbreaks worldwide (21, 66). Symptomatic infection with these strains has been positively associated with secretor status (expression of the FUT2 gene) (98). Novel GII.4 variants emerged over the past 20 years to cause epidemic disease. As discussed above, it is possible that the development of immunity in the population may drive the evolution of these viruses (99, 100).

Transmission

NV is highly infectious, and infections spread rapidly. The human infectious dose 50% has been calculated to be ∽1320 genomic equivalents (virions) in genetically susceptible persons from human experimental infection studies, with as few as 200 genomic equivalents resulting in symptomatic infection (101). Peak virus shedding in feces is as high as 10^{12} genome equivalents per gram, and in vomitus it has been measured to be as high as 10^{7} per mL (101, 102). In semi-closed communities or in volunteer studies, illness attack rates are high (>50% to as high as 90%) and patients present with explosive diarrhea and vomiting. There often is substantial spread to secondary contacts (>50% attack rate), and these characteristics may necessitate the closure and disinfection of hospital wards, cruise ships, or hotels (103). Transmission occurs largely by the fecal-oral route, and exposure risks decrease as sanitary conditions improve and population density decreases. However, transmission by the fecal-oral route alone does not fully explain the rapid spread of these infections. Increasing evidence suggests that some outbreaks have been due to airborne or fomite transmission (103, 104). Proximity to projectile vomiters has been identified as a risk factor, and aerosolized viral genomes have been detected in concentrations up to 2 x 10^{3} genomic equivalents per m^{3} from air samples taken from symptomatic patients' rooms and surrounding hallways and nursing stations in health care facilities (105).

An unresolved question related to transmission is the duration that an affected individual is infectious. The duration of symptomatic illness in healthy adults is 48 to 72 hours. Sensitive antigen detection and RT-PCR assays have shown that excretion occurs in >90% of ill volunteers, shedding is detected in asymptomatic individuals, and antigen shedding starts as early as 15 hours after infection, before symptomatic illness in approximately one quarter of subjects, and is detectable in fecal samples for several weeks after infection (85, 106, 102). Correspondingly, epidemiologic studies confirm transmission of NoVs in association with presymptomatic (107) and postsymptomatic (108) infection.

PATHOGENESIS

In early volunteer studies with NV and SMA, the incubation period ranged from 10 to 51 hours and 19 to 41 hours, respectively (109, 110). Illness usually lasted 24 to 48 hours. In adult volunteer studies where proximal intestinal biopsies were taken (111, 112), histologic changes were seen in jejunal biopsies from ill subjects (Fig. 4). Symptomatic illness was correlated with a broadening and blunting of the intestinal villi, crypt cell hyperplasia, cytoplasmic vacuolization, and infiltration of polymorphonuclear and mononuclear cells into the lamina propria, but the mucosa itself remained intact. Histologic changes were not seen in the gastric fundus, antrum, or colonic mucosa (112) or in convalescent phase biopsies. The extent of small intestinal involvement remains unknown because studies have only examined the proximal small intestine and the site of virus replication has not been determined. Intestinal biopsies from NoV-infected children who were small intestinal transplant recipients showed increased enterocyte apoptosis and inflammation that was difficult to distinguish from allograft rejection (113). NoV infection also is associated with epithelial barrier dysfunction (114). Studies of GII.4 infection in gnotobiotic pigs demonstrated patchy infection of duodenal and jejunal enterocytes, with a few ileal enterocytes also infected (55).

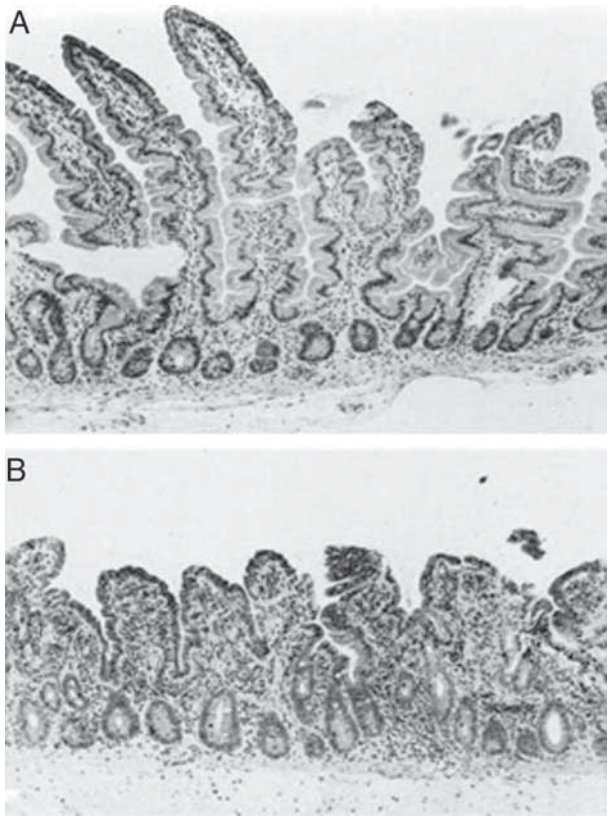

FIGURE 4 Histologic alterations observed in a volunteer after challenge with Norwalk virus. Jejunal tissue from biopsies of a volunteer prior to challenge (A) and after challenge (B) with Norwalk virus. The villi are broadened and flattened during Norwalk virus gastroenteritis illness. (H&E, x90.) From Agus et al. (111), with permission.

Clinical studies of experimental human infection also show that small intestinal brush-border enzymatic activities (alkaline phosphatase, sucrase, and trehalase) are decreased, resulting in mild steatorrhea and transient carbohydrate malabsorption (111). Jejunal adenylate cyclase activity is

not elevated (115) and gastric secretion of HCl, pepsin, and intrinsic factor is temporally associated with these histologic changes. In contrast, gastric emptying is delayed (116). It has been suggested that reduced gastric motility may be responsible for the nausea and vomiting associated with this gastroenteritis. Another hypothesis is that symptomatic illness is due to the host's innate immune response to the viral infection.

Virus Shedding and Extra-intestinal Spread

Fecal viral loads are significantly higher among symptomatically infected persons compared to those with asymptomatic or subclinical infection based upon epidemiological and human experimental infection studies (101, 117, 118), although there is considerable overlap between the two groups. Other factors can also influence the apparent viral load, including the assay used (which can vary in amplification efficiency based upon virus genotype), patient age, and clinical setting (119). Thus, there is not a clear viral load cut-off level that distinguishes between symptomatic and asymptomatic infection. The duration of NoV fecal shedding is similar following asymptomatic and symptomatic infection in otherwise healthy persons (101, 117).

Viral RNA can be detected in the blood of up to one third of young children with NoV gastroenteritis (120, 121). The presence of viral RNA in the blood is associated with higher viral fecal loads and longer hospitalizations for gastroenteritis (120). Viral RNA can be detected in the bloodstream of children with primary immune deficiencies and in NoV-infected adult stem cell transplant recipients (122, 123), but it has not been identified in immunocompetent adults (124). NoV RNA has also been identified in cerebrospinal fluid (123, 125). The clinical importance of the extra-intestinal NoV is unclear at this time.

Immunity and Immune Responses

Adaptive immune responses play a role in protection from NoV and SaV infection in addition to the innate resistance to infection observed from failure to express HBGAs on the gut epithelium. The contribution of adaptive immunity was difficult to discern in early studies because of the uncharacterized involvement of genetic resistance mechanisms. Nevertheless, these studies found that at least 50% of adult volunteers became ill following administration of NV (GI.1) or Hawaii virus (HV, GII.1) (110). Short-term homologous immunity developed based upon the results of early challenge studies in which volunteers who became ill following an initial NV challenge failed to become ill on rechallenge with the same agent 6 to 14 weeks later (97). In several volunteer studies elevated pre-existing levels of serum or intestinal antibody to NV did not correlate with resistance to illness; instead, higher antibody levels were associated with increased susceptibility to illness (96, 97). In contrast, short-term resistance to infection induced by prior homologous infection correlated with antibody levels in other challenge studies, and a correlation between the level of serum antibody and protection has been observed in epidemiologic studies (96, 110, 126).

The recognition of genetic resistance to infection and illness facilitated the assessment of adaptive immune responses. Although serum antibody levels measured by ELISA do not predict susceptibility to illness in an experimental human infection model, higher levels of pre-existing serum antibody that blocks virus binding to HBGAs are associated with a decreased risk of illness in secretor-positive individuals administered NV (127, 128). These findings have also been noted in a GII.4 challenge study (129) and have been proposed as surrogate for neutralizing antibody and as a correlate of immunity. Infection leads to an increase in circulating, virus-specific, IgG- and IgA-producing plasmablasts 1 week later, and as these cells die off, virus-specific memory B cells are generated. Higher levels of IgG memory B cells at the time of virus exposure are also associated with a lower risk of illness (130). Infection also leads to mucosal immune responses that are measurable in saliva and feces. Higher levels of pre-exposure salivary IgA have also been associated with a lower risk of illness, while higher levels of fecal IgA were associated with lower peak levels of fecal virus excretion (130). T cells responses occur after infection, but their relative importance is less well characterized (20). The contribution of past infection with heterologous strains to protection from infection also remains to be determined, as heterotypic HBGA-blocking responses can be observed in both adults and young children (19, 20, 131).

Few studies have examined immunity to SaV infection in young children. One study that measured immunity to SaV/Sapporo/82 using a RIA with hyperimmune antiserum that measured type-specific antibodies found that the presence of serum antibody was clearly correlated with resistance to illness but not to infection (132).

CLINICAL MANIFESTATIONS

The hallmark of infection with NV and other HuCVs is the acute onset of vomiting or diarrhea or both. No prodrome is seen, and the spectrum of illness may vary widely across individual patients. For example, in adults infected with the same experimental inoculum, one volunteer vomited 20 times and required parenteral fluid therapy, whereas a second volunteer had no vomiting but eight diarrheal stools (3). The relative frequency of these and other symptoms in adults experimentally infected with NV (85) (Table 4) is similar to those seen in natural outbreaks and in infection with related viruses (110). Of 50 volunteers orally administered NV, 41 (82%) became infected; of these infections, 68% were symptomatic and 32% were asymptomatic. The most

TABLE 4 Response of 50 adult volunteers (19 to 39 years old) administered Norwalk virus

Response	% of Infected volunteers (N=41)*	% of Uninfected volunteers (N=9)
Seroconversion	98	0
Antigen excretion	88	0
Infection	100	0
Asymptomatic	32	0
Symptomatic	68	0
Symptoms with clinical illness (N=28)		
Diarrhea	86	0
Vomiting	57	0
Nausea	96	10
Abdominal cramps	96	0
Headache/bodyache	96	40
Chills	36	0
Fever (>37.8°C)	32	0

Data from Graham et al. (85).
*Infection determined by antigen shedding and/or antibody response.

common symptoms with clinical illness are nausea, malaise, and abdominal cramps (Fig. 5). Diarrhea, which is usually watery without mucus, blood, or leukocytes, occurs in most patients, and vomiting is seen in most. Subjective or documented fever and chills occur in a minority of patients. The illness is generally mild and self-limited, with symptoms lasting 12 to 48 hours, and illnesses caused by the different NoVs are clinically indistinguishable.

More severe disease can be seen in certain populations. Illness lasts longer in children <1 year of age and in

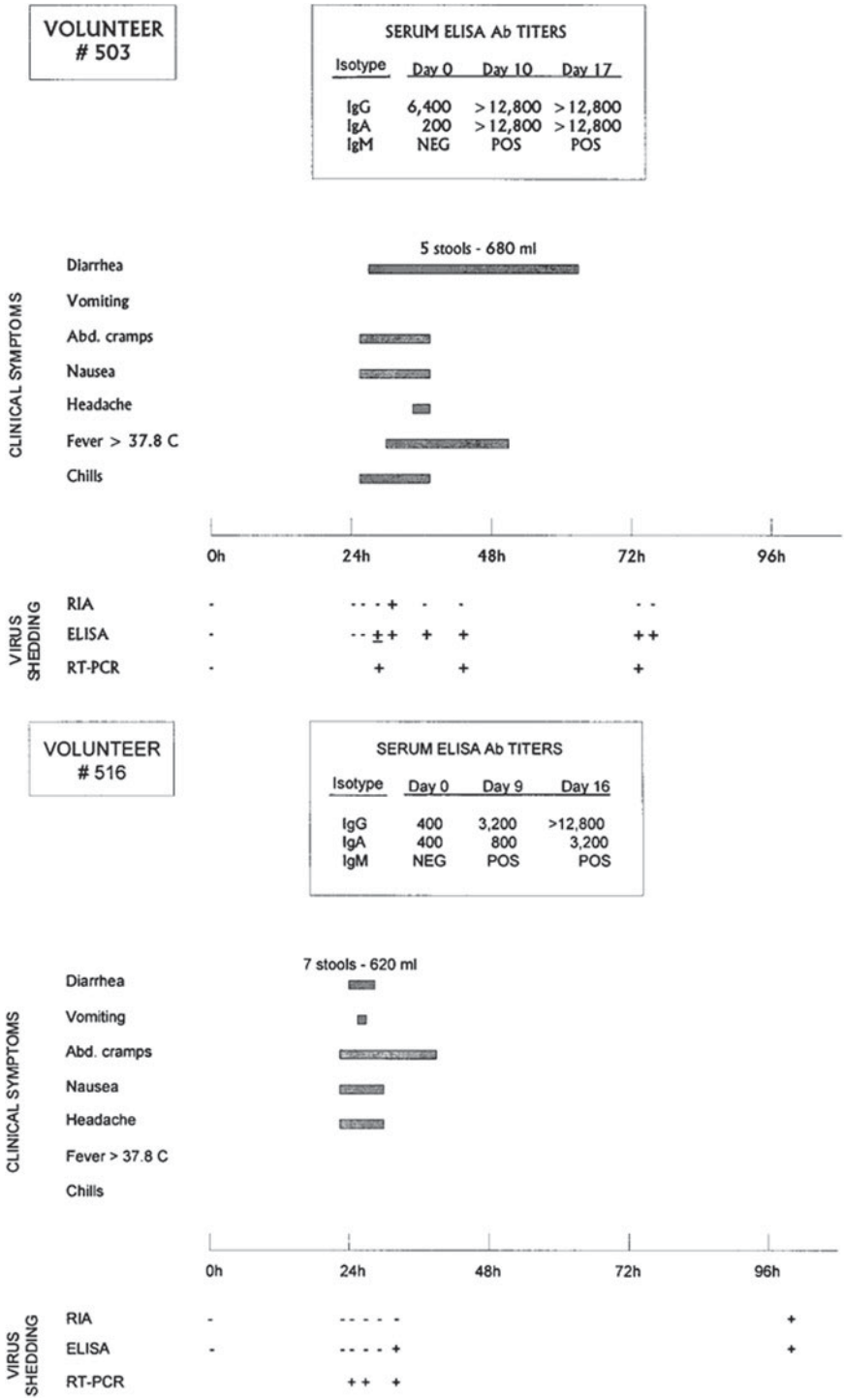

FIGURE 5 Clinical outcome of infection with NV in two volunteers. Clinical course of two volunteers who became ill after challenge with 8fIIa NV inoculum (at 0 h). Both volunteers were considered to have severe disease. Volunteer 503 was a 29-year-old man and volunteer 516 was a 23-year-old woman (85).

TABLE 5 Comparison of methods to detect HuCVs.

	Method	Specificity	Sensitivity	Comments
Detection of viral antigen	Electron microscopy	Depends on operator experience	10^6 to 10^7 pp/mL*	Detects viral particles—least sensitive and specific method available; identifies multiple pathogens
	Immune electron microscopy	Depends on antiserum used	10^5 to 10^6 pp/mL	Detects viral particles
	Enzyme immunoassay	Genogroup-specific	Particulate and soluble antigen 0.025 ng protein	Detects viral particles and soluble antigen; low sensitivity and has limited utility for application to individual patients; good for outbreak identification when multiple samples available for analysis
	Immunochromatography	Genogroup-specific	∽10^4 genomes	Detects viral particles and soluble antigen; similar limitations as enzyme immunoassay, but more rapid result; not currently commercially available in the United States
Detection of genomic RNA	Hybridization	Probes determine specificity	∽10^4 genomes, if no inhibitors present	Detects genome; more labor intensive than other molecular methods
	RT-PCR	Primers and probes determine specificity	10–40 genomic copies, if no inhibitors present	Need several primer sets and probes to detect all HuCVs; can sequence amplicons from conventional RT-PCR assays to characterize virus; real-time assays allow rapid diagnosis; multiplex assays (also detect other enteric pathogens) commercially available
Detection of seroresponses	IEM	Type-specificity depends on antigen used	Not known, but less sensitive than EIAs using rVLPs	Labor-intensive; rarely used
	EIA	Cross-genotype responses occur	Sensitivity depends on match of antigen with infecting strain	VLPs can be used as antigen; requires paired sera; limited to research laboratories

*pp/mL = physical particles per mL.

hospitalized patients (133, 134, 135). Volume depletion with renal insufficiency and hypokalemia are more common in the elderly and in persons with underlying disease (e.g., cardiovascular, immunocompromised)(136). Disseminated intravascular coagulation developed as a complication of NoV infection in a group of previously healthy soldiers exposed to severe environmental stress (137). Chronic diarrhea lasting months to years in association with continued viral shedding can be seen in immunocompromised patients (87, 113). Most complications are associated with volume depletion or aspiration of vomitus and can include death, especially in the elderly. Benign infantile seizures have occurred in young children with NoV gastroenteritis, although a causal relationship remains to be established (138).

A provisional diagnosis of infection during outbreaks of gastroenteritis is possible if the following criteria are met: (1) absence of bacterial or parasitic pathogens; (2) vomiting in more than 50% of cases; (3) mean (or median) duration of illness ranges from 12 to 60 hours; and (4) an incubation period of 24 to 48 hours. These criteria were met in 81% to 100% of ill individuals in 38 NoV outbreaks, although more recent analyses of the application of the criteria to food-borne outbreaks investigated by the CDC yielded a sensitivity of 68% (139, 140). A definitive diagnosis, desirable for both clinical and epidemiological studies, requires the use of a detection method for antigen, the viral genome, or antibody responses.

LABORATORY DIAGNOSIS

Because HuCVs cannot be grown in cell culture initial assays for viral diagnosis were developed using reagents (pre- and post-infection serum and stool) from volunteer studies (reviewed in (15)). The first specific test developed for NV diagnosis was IEM (2), and this method is still used for the examination of fecal samples for the presence of NV and other HuCVs in some diagnostic laboratories. However, laboratory diagnosis of HuCV infections has changed with the sequencing of NV and subsequent expression of the NV capsid protein (Table 5), and molecular diagnostics have become the gold standard for diagnosis (7). Recombinant VLPs for NoVs and SaVs also have been used to develop diagnostic assays described below. The use of some of these assays remains restricted to research laboratories.

Nucleic Acid Detection

The detection of viral nucleic acids has become the preferred strategy for identifying NoV and SaV infection because of the high sensitivity of this approach. A number of different assay types are currently used for virus detection, although the availability of these assays varies across different countries and clinical settings. RT-PCR-based assays are the most commonly used molecular tests, although isothermal amplification methods such as nucleic acid sequence-based

amplification (NASBA) and loop-mediated isothermal amplification (LAMP) and hybridization-based assays have also been described. Among the RT-PCR-based assays, both traditional RT-PCR and real-time RT-PCR assays are used, with the latter providing more rapid results. An automated sample preparation and real-time RT-PCR assay system (Xpert Norovirus Assay [Cepheid]) has received FDA approval (141). Multi-pathogen assays that identify bacterial, viral, and parasitic pathogens in fecal samples of persons with gastroenteritis are also now commercially available (Luminex xTAG® Gastrointestinal Pathogen Panel [Luminex], FilmArray™ Gastrointestinal Panel [Biofire Diagnostics, LLC]) (142).

Several factors can affect the sensitivity and specificity of RT-PCR assays (15). Clinical samples and environmental samples may contain inhibitory substances that prevent amplification of viral RNA. A variety of methods have been developed to purify viral nucleic acids from these samples, and these methods vary in their ability to remove inhibitors successfully. A strategy for detection of the presence of inhibitors is the use of an internal standard RNA that is amplified only in the absence of significant amounts of inhibitor. Primer and probe selection also impacts assay performance. A number of different primer pairs and different RT-PCR conditions have been described for the detection of NoVs and SaVs (8, 13, 15, 143). No single primer pair will detect all NoVs or SaVs, and no assay has been universally accepted as a standard. Several different conserved regions of the genome have been targets for nucleic acid amplification (viral polymeraseVP1, capsid-specific, and NTPase), but recognition that the ORF1-ORF2 junction is highly conserved in NoVs has made this a prime target for amplification (143). Because no single primer pair detects all NoVs or SaVs, two or more primer pairs must be used to have a reasonable likelihood of virus detection.

RT-PCR assays are most commonly used to detect virus in stool samples, but they also have been used to detect virus in vomitus and throat swabs (15). RT-PCR assays are being used increasingly to detect NoVs in foods (primarily shellfish), water samples, sewage, and other environmental samples (e.g., swabs collected from areas potentially contaminated by ill persons) associated with outbreaks of viral gastroenteritis (15, 82). The potential utility of these methods for food and water safety is under evaluation.

Antigen Detection

The first immunologic assays developed to detect antigen and amenable to large-scale application were radioimmunoassay (RIA) and subsequently enzyme immunoassays (EIAs) (15). Human volunteer pre-infection and convalescent serum were initially used as capture and detector antibodies in these assays, but these antibody reagents have now been replaced with hyperimmune and monoclonal antisera made to rVLPs. Hyperimmune antiserum to rNV VLPs produced in guinea pigs, mice, and rabbits reacts with high titer to the immunogen and to native NV in stool (25, 76, 85). Hyperimmune antisera were used to develop an antigen EIA that has been shown to be highly sensitive and specific for NV antigen (85, 144). The test sensitivity of the standard antigen EIA was determined to be ~0.025 ng of capsid protein (1.4×10^6 virions) and antigen was detected in the stools of volunteers at dilutions as high as 1:10,000 (85). The rNV antigen EIA detects both virus particles and soluble protein in clinical samples. Antigen detection assays using hyperimmune antisera generated against a single virus genotype have been found to be sensitive and specific for viruses belonging to that genotype, but have had limited utility due to their type specificity.

Cross-reactive epitopes that are shared within a NoV genogroup or across genogroups have now been identified, and monoclonal antibodies that detect GI-specific or GII-specific epitope have been used to produce more broadly reactive antigen rEIAs (145). These assays have been commercialized outside of the United States in a variety of assay formats, and one kit has been approved for use in the United States for identification of norovirus outbreaks (7, 146, 147). However, improvements in sensitivity and specificity are needed before these tests are useful as a diagnostic for the individual patient, in contrast to application to outbreak investigations where multiple samples are available (148).

Serologic Assays

The use of serological assays for diagnosis requires paired serum samples to allow identification of a change in antibody level. Significant rises in virus-specific IgA and IgG titers can be detected as early as 7 days after infection (101). Virus-specific IgM antibody also can be detected within two weeks of infection. The use of serological assays has generally been restricted to research laboratories.

EIAs to detect antibody responses have been developed using rVLPs as the antigen (15, 21, 25, 85). Total, class, or subclass-specific serum antibodies, can be detected depending on the reagents used to detect the bound human antibody. The assay can be modified to detect virus-specific IgA in fecal samples or to detect virus-specific IgM using an antibody capture EIA format.

As noted earlier, antibody that blocks binding of VLPs to HBGAs has been proposed as a surrogate for NoV neutralizing antibody since culture systems have not been available for these viruses (149). Although increases in HBGA-blocking antibody occur after infection, the magnitude of antibody response (i.e., fold-rise) is usually lower than that seen using a standard ELISA.

Heterologous antibody responses can often be detected in subjects following infection (e.g., antibody rise to GII.2 SMA following infection with GI.1 NV) (19, 21, 150, 151). A heterologous response only appears to occur if a homologous antibody response also has occurred, and the heterologous response is of a lower magnitude than that of the homologous response (e.g., 4-fold rise in antibody titer versus a 16-fold rise) (151). Heterologous responses involving IgM or IgA antibody occur infrequently (150, 151).

PREVENTION

No specific methods are available for the prevention of HuCV infection or illness. Because these agents are highly infectious, handwashing and disposal or disinfection of contaminated material may decrease transmission within a family or institution. Special care must also be given to the hygienic processing of food in view of the frequent occurrence of food-borne outbreaks of NoV infection. Ill food handlers should not prepare food for a minimum of three days after their illness, and plastic gloves should be worn to prepare foods. Consumption of raw shellfish is a risk, since outbreaks have occurred from the consumption of shellfish that meet current microbial (bacterial) sanitary standards. Human breast milk from secretor positive women contains fucosylated mucins that block binding of some NoV strains to HBGAs expressed on epithelial cells and may play a role in protecting infants from NoV infection (152, 153).

Immunoprophylaxis

Vaccines are being developed to try to prevent norovirus infection and illness (66). A vaccine based upon using a NoV VLP as an immunogen is the only candidate that has been in human clinical trials. This vaccine is immunogenic when delivered as a monovalent vaccine intranasally or as a bivalent vaccine delivered intramuscularly (129, 154). Both routes of administration have led to a modest level of vaccine-induced protection following experimental virus challenge administered shortly after completion of the two-dose vaccine regimen, decreasing illness or illness severity (128, 155). These vaccine studies have been conducted in adults, and many additional studies in other populations (e.g., pediatric, elderly, immunocompromised) along with field efficacy studies are needed to determine the potential utility of a vaccine strategy for prevention of illness or infection. That a vaccine strategy could prove effective is suggested by the short-term immunity to reinfection with NV observed following repeated experimental challenge, the apparent widespread and broad immunity to the SaVs after childhood, and by the correlation of higher serum HBGA-blocking antibody levels with protection from illness and infection (96, 97, 127, 129). The impact of virus diversity, antigenic drift, past infection history, and waning immunity on vaccine responsiveness and effectiveness still need to be determined. The development of HBGA-blocking monoclonal antibodies brings the potential for their use in passive immunoprophylaxis, although the utility of this approach still needs to be assessed (156).

Management of Outbreaks

Outbreaks can result in significant morbidity and economic loss because of frequent secondary transmission of diseases. Outbreaks are a particular concern in closed environments such as hospitals, nursing homes, or ships because all personnel can rapidly become disabled. Virus can persist in the environment and lead to recurrent outbreaks (95). Ill persons should be isolated and handwashing and disposal or disinfection of contaminated material put into effect immediately. Control measures for outbreaks of viral gastroenteritis should focus on the removal of an ongoing common source of infection (e.g., an ill food handler or the contamination of a water supply), adequate disinfection of contaminated areas (e.g., with a sodium hypochlorite-based or other EPA-approved disinfectant), and on the interruption of person-to-person transmission that can perpetuate an outbreak in a population after the common source has been removed (157, 158).

The facts that asymptomatic infection occurs more frequently than previously realized and that antigen may be shed for 2 to 3 weeks after exposure need to be recognized in managing such outbreaks. This is particularly important in instituting and overseeing the hygienic processing of food for and care of the elderly. The potential for contaminated water sources should be assessed based upon the outbreak setting, and possible sources must be eliminated. Methods for outbreak management will probably improve as new tests are used in epidemiological studies of virus transmission in various settings. For example, if asymptomatic food handlers or hospital staff can be identified, they may be furloughed until they are no longer excreting virus and potentially infectious. Because improvements in environmental hygiene may not be accompanied by reductions of endemic diarrhea caused by viruses, immunization may play an important role in future control.

TREATMENT

As discussed above, the illnesses caused by these viruses are generally mild and self-limited, and resolution occurs without sequelae (133). Hospitalization for rehydration is occasionally required, but the major impact of this disease has been morbidity and loss of time from work and school. Treatment involves symptomatic therapy, with oral rehydration generally being sufficient. In rare cases, parenteral administration of intravenous fluids is required. Antiemetics and antidiarrheals may provide some relief of symptoms, although no controlled studies have been performed to demonstrate the efficacy of such treatments in HuCV acute gastroenteritis. Deaths from NV gastroenteritis have been reported in the elderly and from HuCV infections in immunocompromised children, although some of these have been attributable to other primary causes. The use of human milk or oral immunoglobulin therapy anecdotally has not had any therapeutic efficacy in an immunocompromised patient (159). Currently no antiviral drugs exist to treat the caliciviruses, but this may change once these viruses are successfully propagated or with the recent availability of high-resolution structures of several of the viral proteins that have enzymatic activities.

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Hepatitis E Virus

HARRY R. DALTON, NASSIM KAMAR, AND JACQUES IZOPET

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Hepatitis E was recognized as a disease in 1980 following a large outbreak of unexplained hepatitis in Kashmir, India, in 1978. The outbreak affected 52,000 individuals, mostly young adults, with a self-limiting hepatitis similar to hepatitis A (1). However, this was not hepatitis A, because there was a very high mortality rate in afflicted pregnant women. Further, most of those infected had previously been exposed to HAV (2). In 1983, the causative virus was discovered by electron microscopy in the stool of a scientist from the former USSR who drank a pooled fecal extract from Soviet troops with unexplained hepatitis serving in Afghanistan (3). The viral genome was sequenced and given the name HEV in 1990 (4). Many of the historical outbreaks of unexplained hepatitis in Asia and other developing areas were subsequently found in retrospect to be caused by HEV (5, 6).

For over 20 years, hepatitis E virus (HEV) was considered a disease that was mainly endemic in developing countries and only of clinical relevance in developed countries in travelers. We now know that this concept is incorrect, because locally acquired zoonotic hepatitis E has been found in every developed country where it has been sought. HEV is endemic in many European countries and hyperendemic in a minority. In addition to acute infection, zoonotic HEV causes chronic infection in the immunosuppressed host and is a surprisingly common contaminant of the human blood supply. Current thinking is that HEV is a pathogen of global significance and is probably the most common cause of acute viral hepatitis worldwide (7).

VIROLOGY

Taxonomy

HEV is a member of the *Hepeviridae*, a family that consists of two genera, *Orthohepevirus* and *Piscihepevirus* (8). Only one HEV serotype is recognized. The genus *Orthohepevirus* contains three species that infect mammals (*Orthohepevirus* A, C, and D) and one that infects birds (*Orthohepevirus* B) (Fig. 1). The *Orthohepevirus* A species, which includes strains that infect humans, contains seven genotypes (HEV 1 to HEV 7). HEV1 and HEV2 are found only in humans, whereas the other genotypes infect both human and other animal species, including pigs, wild boar, deer, rabbits, mongoose, and camels. However, the other Orthohepeviruses do not infect humans: B infects chickens, C infects rats and ferrets, while D infects bats. Lastly, the Piscihepeviruses infect the cutthroat trout. Despite the lack of robust criteria, subgenotypes have been identified within the four major HEV genotypes (5 for HEV1, 2 for HEV2, 10 for HEV3, and 7 for HEV4) (9). Complete reference genome sequences are available to facilitate genotyping and molecular epidemiology studies (10). Subgenotypes 1a, 1b, and 1c are prevalent in Asia, while subgenotypes 1d and 1e are found in Africa (9). The HEV viruses found in Europe are genotype 3. The distributions of 3f, 3c, and 3e strains in human and pig populations in France are the same, which indicates zoonotic transmission from this animal reservoir (11). The temporal distributions of subgenotypes 3f and 3c in the UK and France seem to be evolving, with increasing frequency of subgenotype 3c (12, 13).

Virus Structure and Morphology

HEV is a small RNA virus with an icosahedral capsid. Two forms of fully infectious particles have been described. The virions shed in the feces are not enveloped, 27 to 34 nm in diameter, and with a density of 1.27 g/cm^3. The virions circulating in the blood are cloaked in host cell membranes; their density is 1.15 g/cm^3 (14). The host-derived membranes protect the virus from neutralization by antibodies and may play an important role in cell tropism (14, 15).

Genome Organization and Proteins

The HEV genome is a single-strand, positive-sense RNA of approximately 7.2 kb. It consists of a short 5′ noncoding region that is capped with 7-methyl-guanosine; three open reading frames (ORFs), ORF1, ORF2, and ORF3; and a short 3′ noncoding region that ends in a poly-(A) tail (Fig. 2).

ORF1 encodes a non-structural protein about 1700 amino acids long that is involved in HEV RNA replication. This protein contains several functional domains: a methyltransferase/guanyltransferase, cysteine protease, macrodomain (X domain), RNA helicase that has 5′-nucleoside triphosphatase activity, and an RNA-dependent RNA polymerase (16). A variable region encoding a proline-rich hinge, the polyproline region (PPR), is an intrinsically disordered region in which segments of human genes have been identified (17–22). Immunocompromised patients who develop a chronic infection have a highly heterogeneous mixture of PPR quasispecies at the acute phase of HEV

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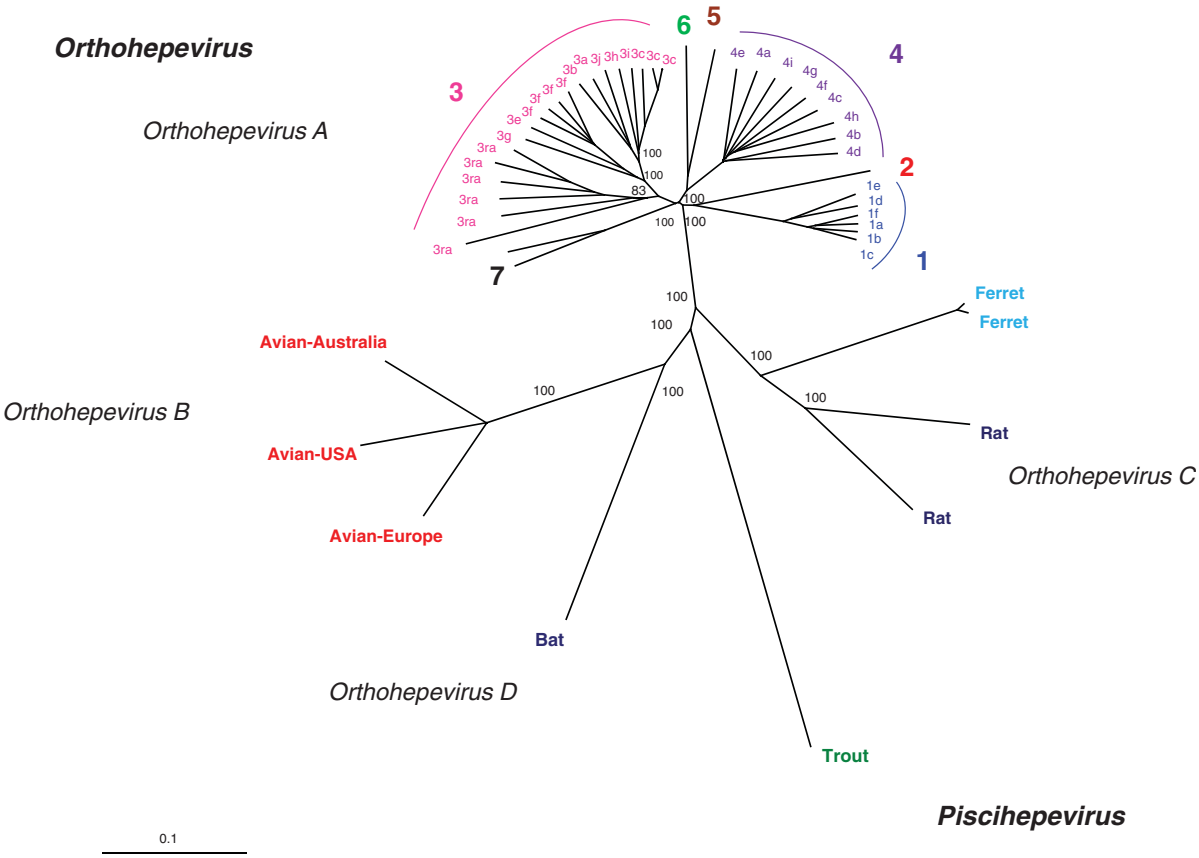

FIGURE 1. Family *Hepeviridae*—Phylogenetic tree based on full-length sequences of HEV strains (neighbor joining method and bootstrap analysis).

infection (23). The ORF1 of HEV1 includes a novel OFR, ORF4 (24). ORF4 is expressed after endoplasmic reticulum stress, and interacts with eukaryotic elongation factor 1 isoform-1 (eEF1α1), so stimulating virus polymerase activity.

ORF2 encodes the 660 amino acid virus capsid protein. This protein has three glycosylation sites, Asn 132, Asn 310, and Asn 562 and an N-terminal signal peptide that drives its translocation into the endoplasmic reticulum (25). The capsid protein has been divided into three domains: shell (S), middle (M), and protruding (P) domains. The P domain is the major target for neutralizing antibodies and contains a putative receptor binding domain (26, 27). Capsid monomers self-assemble to form dimers and then the decamers that encapsulate the virus RNA. The capsid consists of 180 copies arranged as an icosahedron with T=3 symmetry (28). Immunological and structural studies of the capsid protein have contributed to the development of a hepatitis E vaccine (29, 30). Immunocompromised patients whose HEV infection becomes chronic are infected with HEV quasi-species whose genome is highly heterogeneous in regions encoding the M and P domains of the capsid protein (31).

ORF3 encodes a small protein, 113 residues in HEV3 and 114 residues in HEV1, 2, and 4, that is essential for virus egress. ORF3 protein is associated only with enveloped HEV particles, not with naked HEV particles. The ORF3 protein must be phosphorylated on Ser80 before it can interact with the non-glycosylated form of the capsid protein (32). The ORF3 protein contains a conserved PSAP motif that enables it to interact with the endosomal sorting complex required for transport (ESCRT), including the tumor susceptibility gene 101 (Tsg 101) (33–38).

HEV Replication Cycle

The recent development of several cell culture systems has led to a better understanding of HEV target cells and the virus replication cycle (39, 40).

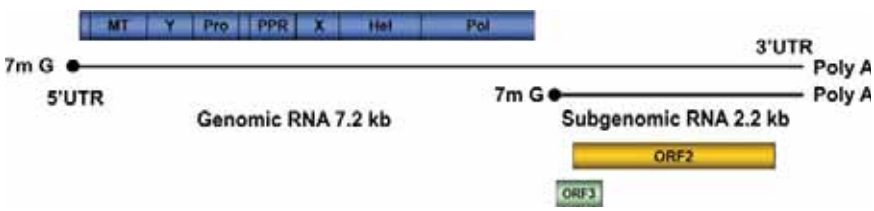

FIGURE 2. Hepatitis E virus genome.

Unenveloped HEV particles attach to the liver cells via heparan sulfate proteoglycans and other potential receptors (Fig. 3) (41). Subsequent events include clathrin-mediated endocytosis involving the dynamin-2 and membrane cholesterol pathways and cytoskeleton remodeling (42, 43). Quasi-enveloped HEV particles are internalized through a clathrin- and dynamin-2 dependent pathway but require (i) Rab5 and Rab7 small GTPases involved in endosomal trafficking, (ii) endosomal acidification, and (iii) lysosomal lipid degradation (44).

The virus RNA is directly translated into ORF1 polyprotein. Whether the polyprotein is cleaved into its separate functional units or functions as a single multidomain protein remains unclear. The viral methyltransferase, protease, helicase and polymerase activities are used to replicate the genomic RNA to give negative-sense RNA intermediates that then serve as templates for the synthesis of genomic and subgenomic positive-sense RNAs. The subgenomic RNA is translated into the ORF2 and ORF3 proteins (45). The positive-sense genomic RNA is packaged into progeny virions.

The tumor susceptibility gene 101 (Tsg101) that interacts with the ORF3 protein and the enzymatic activities of vacuolar protein-sorting protein 4 (Vps4A and Vps4B) are both involved in the release of virions from infected cells (36). HEV particles may acquire a membrane when they are released with internal vesicles from the multivesicular body (MVB) via the cellular exosomal pathway (35). Bile could degrade the membrane through its detergent action.

Inactivation by Physical and Chemical Agents

Cell culture and *in vivo* experimental models show that inactivation of HEV in food products derived from infected pork liver is achieved after a cooking time of at least 20 min at an internal temperature of 71°C (46, 47), indicating that infectious HEV can persist after rare and medium-to-rare cooking. Viable HEV has been found in a range of pork products in the human food chain in many countries, and this has led to the proposition that a key route of infection in humans is via consumption of undercooked or uncooked pork. Many pork products are prepared for consumptions by air-drying and/or curing. It is unknown if these processes inactivate HEV. Such products are potentially an important source of HEV infection in humans because they are mostly consumed without cooking. Chlorination and UV treatment can reduce HEV in drinking water, but experimental data are still limited (48, 49). Several steps of the manufacturing process of plasma derivatives have been studied by applying HEV inactivation or removal experiments (50). Nanofiltration is effective at removing HEV, ethanol fractionation has limited efficacy, and lowering the pH is ineffective. Liquid heating, like pasteurization, is partially successful at removing HEV but depends on process

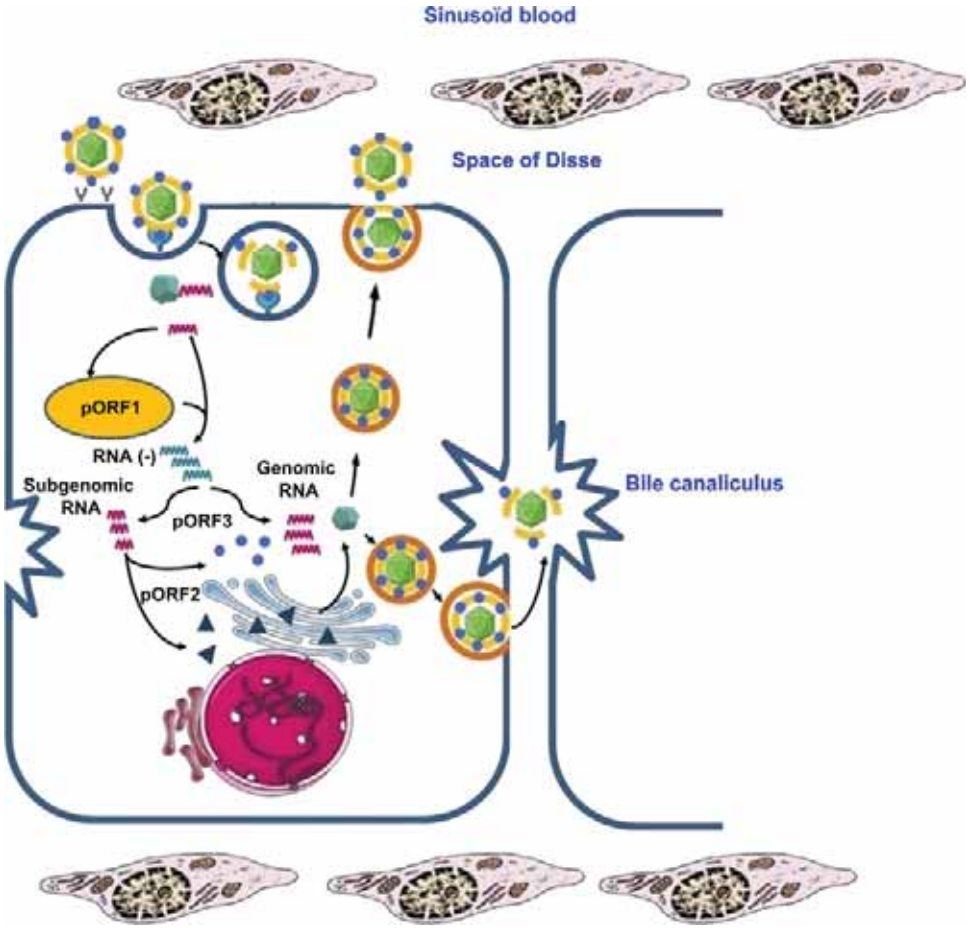

FIGURE 3. Hepatitis E virus replication cycle.

conditions. Treatment by solvent, detergent, or amotosalen does not inactivate HEV.

EPIDEMIOLOGY

Classical Hepatitis E

HEV1 and HEV2 are obligate human pathogens spread primarily by ingestion of infected water. HEV1 is endemic in many parts of Asia, particularly in southern countries of the continent, including India, Pakistan, and Nepal. HEV2 is much less common and is found in some African countries. HEV2 caused a large outbreak of hepatitis E in Mexico in the 1980s, but in recent years there have been few documented cases. HEV1 and HEV2 cause sporadic cases of hepatitis E but are also responsible for large outbreaks involving hundreds or thousands of cases. These occur when there is a breakdown in sanitary infrastructure. Examples of this occurred in Delhi, India in 1953 to 1954 (5) when human sewage contaminated the drinking water, resulting in an outbreak involving over 50,000 cases and the Kanpur, India epidemic of 1991 with over 70,000 people affected (51). Outbreaks of hepatitis E have also been particularly common in African refugee camps over the past 20 years.

Direct person-to-person spread of HEV1 and HEV2 is less important than waterborne spread, because secondary cases are rarely seen in household contacts of hepatitis E cases. In Africa, HEV1 and HEV2 are widely distributed and cocirculate in some populations. Both genotypes have been recorded from outbreaks among displaced people. Waterborne transmission is undoubtedly an important route of infection in such cases, often exacerbated by heavy rainfall. However, data from an outbreak of HEV1 infection in Ugandan refugee camps suggest that intra-household transmission can occur (52).

During an outbreak of hepatitis E the clinical "attack rate" (the percentage of infected individuals with clinically evident infection) is variable. This ranges from up to 15% in India (53) to 30% in an outbreak in Uganda (52). Overall, the average clinical attack rate is approximately 20% (54). Previous exposure determines the proportion of the exposed population susceptible to infection, and geographical variations in previous exposure are likely to explain variability in clinical attack rates. However, reinfection can occur when antibody levels fall below a critical level (7, 55). During outbreaks young adult males are most likely to have symptomatic infection. For example, in the outbreak that occurred in Kanpur, India (51), approximately 80% of cases were aged less than 40 years and M:F ratio was 1.7:1. In most cases the illness is self-limiting, but a high mortality is seen in pregnant women and patients with underlying chronic liver disease (7) (see below).

Zoonotic Hepatitis E

For many years hepatitis E in developed countries was regarded as an uncommon imported infection, largely found in travelers returning home from endemic areas of Africa or Asia. Approximately 10 years ago there were increasing reports of cases of hepatitis E in non-travelers mainly from Europe and Japan. These were found to be caused by HEV3 and HEV4 with very close sequence homology to the HEV found in local pigs, wild boar, and deer populations (56). HEV3 and HEV4 are mainly anthropozoonotic and can be found in a very wide range of mammals, but the true primary host is the pig. The animals infected with HEV appear to have no symptoms. HEV3 is found worldwide, but HEV4 is geographically limited to East Asia, mainly China and Japan, and more recently documented in both pigs and humans in Europe. In contrast to HEV1 and HEV2, large outbreaks of hepatitis E caused by the zoonotic genotypes have not been observed and cases are generally sporadic. There have been occasional small clusters of hepatitis E in humans in developed countries, which are caused by common consumption of infected foodstuffs (57).

HEV has been detected in range retail meat (mainly pork) products in many different countries (58–61). Consumption of meat or meat products (including pig liver sausage, boar, and deer meat) contaminated with HEV is a proven and probably principal route of infection, but there are other ways HEV can skip from its hosts to human beings. HEV has been found in shellfish in a number of locations (62–64), and an outbreak on a cruise ship in 2008 was epidemiologically linked to consumption of this foodstuff (65). Direct animal contact may be the source of infection in some cases, because anti-HEV IgG antibodies are more commonly found in veterinarians and pig farmers than in control groups (66, 67). Environmental contamination with animal feces may be another source of infection. This might occur either directly via contaminated water (68) from farm water "run-off" or indirectly via contaminated food crops. The latter possibility is given some plausibility by the detection of HEV in strawberries which had been irrigated with infected water (Fig. 4) (7). A recent study from France has shown that drinking bottled water seems to protect against exposure to HEV (69). These findings suggest that, as in developing countries with HEV1 and HEV2, zoonotic HEV might also be spread via contaminated domestic water supplies. Another recent study from China found HEV4 in cows and their milk; HEV4 contaminating the milk can survive pasteurization (70). This route of infection may be important and deserves further study. However, in most cases of documented hepatitis E, it is not possible to be certain of the route of infection. There is no evidence of sexual transmission and little evidence of person-to-person spread, except via the human blood supply (see below). Transfusion-transmitted infection is thought to cause only a very small minority of human infections, approximately 1% in a recent study of English blood donors and recipients (71).

In contrast to HEV in developing countries, HEV3 and HEV4 tend to affect older males and excess mortality in pregnant women is not seen. In one study from England the M:F ratio was 3:1 and the median age 63 years (72). The finding that older males are most likely to develop clinically apparent acute hepatitis on exposure to HEV3 and HEV4 is a consistent observation, but unexplained. It seems likely that this relates to host factors, rather than differential exposure, because individuals of all ages appear to be exposed to HEV. One possible explanation is that clinically apparent hepatitis is more likely to be evident in patients with subclinical hepatic steatosis/fibrosis. In a study from England some patients with hepatitis E were heavy alcohol consumers and an excess number were diabetic, both of which are risk factors for hepatic steatosis and fibrosis (73).

Seroprevalence

The response to infection includes the production of specific IgG antibodies, which persist and protect against further infection. However, until recently, our understanding of anti-HEV IgG seroprevalence has been problematic because many of the early studies employed serologic assays of very poor sensitivity (74). This led to gross underestimations of

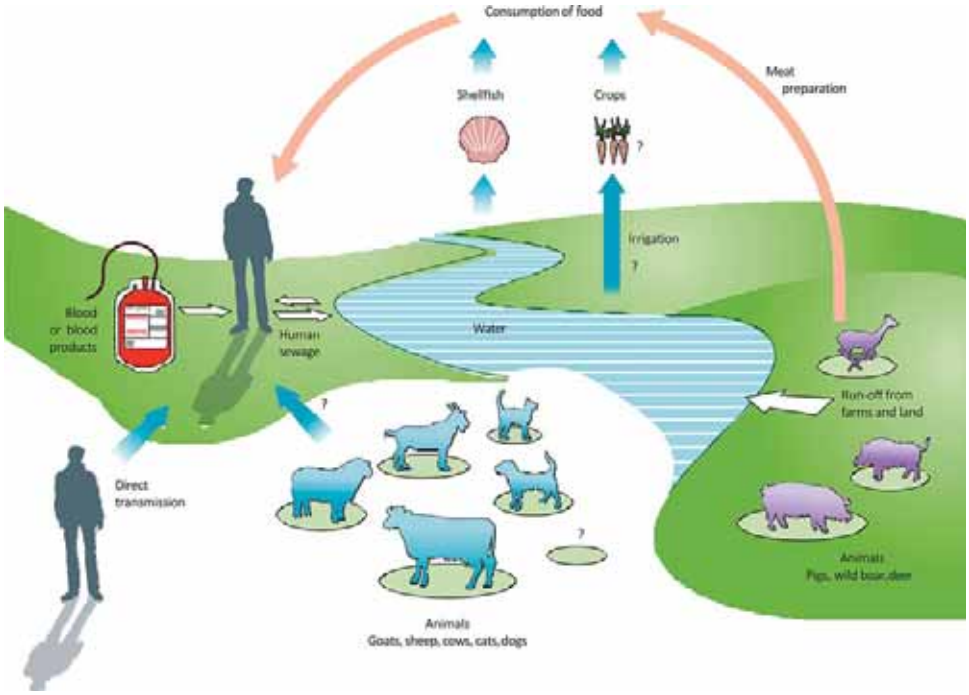

FIGURE 4. Sources and possible routes of infection.

seroprevalence and retarded our understanding of HEV as a pathogen of global significance.

In developing countries, most of the studies are dated and the majority employed assays of poor quality. These studies generally show that seroprevalence is low in children and rises in adolescents and young adults (75). For reasons that are not understood, the situation in Egypt is different, and the seroepidemiology is quite similar to that of HAV: rates of HEV IgG seropositivity rise very rapidly in early childhood and by early adulthood exposure to HEV appears to be nearly universal (76). A recent study from Bangladesh showed that exposure to HEV was found to be an order of magnitude higher when a more sensitive assay is employed (77). Seroprevalence increases steadily from childhood to the age of 40 years, then plateaus in older people at 75% to 80% (Fig. 5). Only in the last few years have we realized that HEV is endemic in developed countries and has been so for generations. One of the key reasons for this delay in our

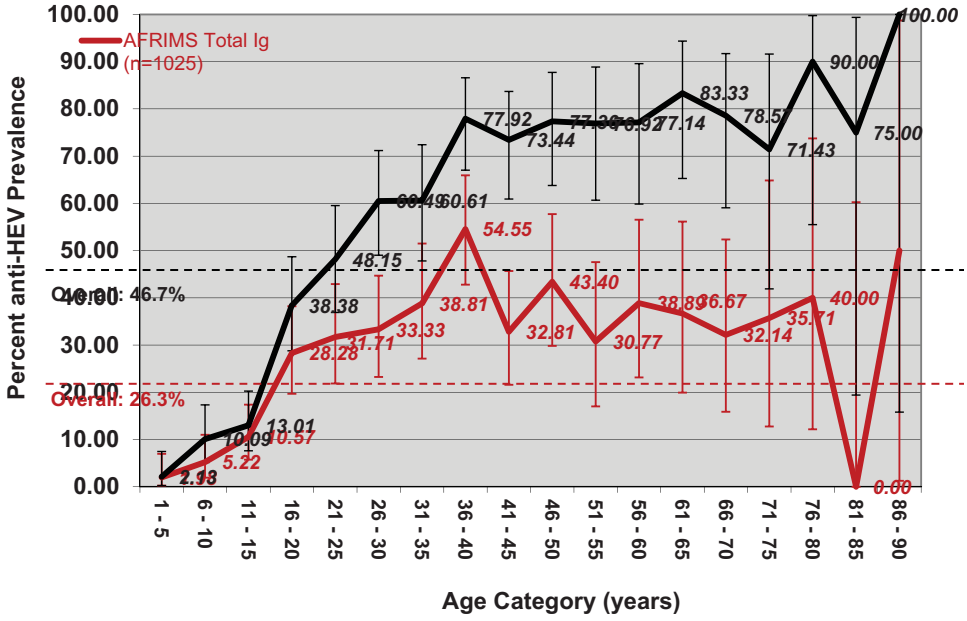

FIGURE 5. Anti-HEV IgG seroprevalence in rural Bangladesh by age. The red line shows the seroprevalence using the AFRIMS assay. The black line shows the seroprevalence (same samples) using a validated Chinese assay, producing seroprevalence estimates an order of magnitude higher.

understanding is that studies from the 1990s showed low (often <5%) seroprevalence rates; anti-HEV IgG positive subjects from developed countries were assumed to be either those who had traveled to endemic areas or had false-positive serology due to cross-reactive antibodies (74). In a Dutch study published in 1993 the anti-HEV IgG seroprevalence was found to be 1% (78). However, using a validated assay of high sensitivity, the seroprevalence from around the same time period was recently found to be 47% (79).

As more recent studies using validated and sensitive anti-HEV IgG assays have been published, the epidemiology of HEV in developed countries has become clearer. In southwest France seroprevalence starts to rise in childhood with a steady increase, which is sustained until old age (80). In western European countries, such as the United Kingdom (81), the Netherlands (79), and Denmark (82), HEV infection was common and seroprevalence rates were high by the mid-20th century. It appears that a large cohort of the population was exposed to HEV in the years of turmoil following the 2nd World War. Toward the end of the 20th century seroprevalence rates in all three countries fell significantly. However, over the last couple of years in the Netherlands seroprevalence has risen quite sharply, particularly in young adults (79). The reason for this recent upswing in seroprevalence is not known.

Seroprevalence also varies between and within countries. Countries with a high seroprevalence include Germany (29%) (83), France (22%) (69), and the Netherlands (21%) (79). In the Netherlands seroprevalence varies little within the regions of the country (84). In contrast, seroprevalence varies significantly in France by region, with values ranging from 8% to 86% (69). Very high seroprevalence rates are found in southern France (Toulouse, Marseille, and Corsica) and in the northeast of the country. In the United Kingdom, the seroprevalence is 12% to 16% in England (72, 81) and 4.5% in Scotland (85). The reason for these differences is uncertain but may relate to differential rates of exposure to HEV due to differences in national and regional culinary cultures.

Incidence

Every year, an estimated 20 million HEV infections occur in developing countries, resulting in over 3 million clinical cases and 70,000 deaths (54). This figure of the "Global Burden of disease" is an underestimate of the true impact of HEV in developing countries for two reasons. First, it includes data from only a limited number of developing countries. Second, the calculations were based, at least in part, on seroprevalence data from studies that employed insensitive assays as shown in Figure 5 from Bangladesh (77).

In developed nations, the incidence of locally acquired hepatitis E varies between and within countries and over time. In the United Kingdom the annual incidence has been estimated at 0.2% (81), the Netherlands 1.1% (84), and in Southern France 2% to 3% (69). In the French national study of HEV, incidence mirrored seroprevalence and was found to be significantly higher in southern and northeastern France (69). In most European countries, particularly in western Europe, the number of laboratory-confirmed cases has increased rapidly over the last few years (Fig. 6) (86). While some of the observed increase can be accounted for by improved case ascertainment, it appears that there has been a true increase in incidence in France, England, and the Netherlands (69, 71, 79). The most dramatic increase has been in the Netherlands, where the number of viremic blood donors has increased from 1 in 2,671 in 2011 to 2012 (84) to

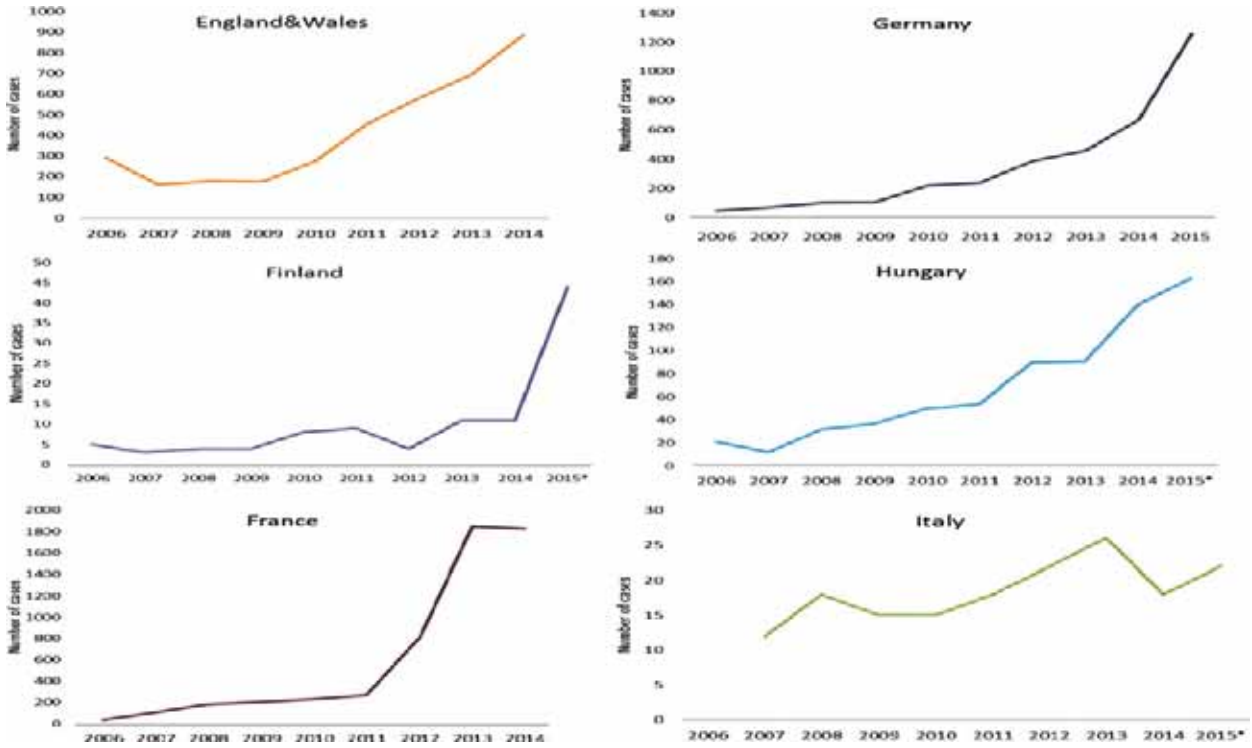

FIGURE 6. Laboratory-confirmed cases of HEV in several European countries. The vast majority of infections were locally acquired and caused by HEV3.

1 in 600 in 2014 (87), accompanied by rapidly increasing seroprevalence rates in young adults (79).

The incidence of hepatitis E has also increased recently in the United Kingdom, and it is currently thought that there are at least 100,000 infections per year, many of which are asymptomatic (71). The increased incidence relates, in part, to improved case ascertainment. However, this has been accompanied by a true increase in incidence (see below). This increase has been accompanied by a change in the strains of HEV recovered from human cases. Historically, HEV RNA causing human disease in the United Kingdom has had very close sequence homology to HEV RNA found in the local pig population. In recent years, coinciding with increased incidence, HEV RNA recovered from human cases in the United Kingdom now bears much more similarity to HEV found in pigs from the Netherlands and Denmark. The explanation for this observation is unclear, but it suggests that there has been a significant and recent increase in HEV contamination of the pork food chain (12). Targeted blood donor screening for HEV was introduced in the United Kingdom in March 2016. Initial, and as yet unpublished, results show a dramatic increase in the number of viremic blood donors, most marked in Scotland. Previously the seroprevalence in Scotland was found to be very low, so currently there appears to be large amounts of circulating virus in a largely HEV-naïve Scottish population. There are a number of other "hot-spots" with high incidence and seroprevalence rates in Europe (Fig. 7).

PATHOGENESIS

Several animal models of HEV infection have been developed (88). Chimpanzee, rhesus macaques, and cynomolgus macaques have been most frequently used in pathogenesis and vaccine studies. These models are adequate for all HEV genotypes. Using a low viral inoculum, cynomolgus macaques can be infected without alanine aminotransferase elevation (89). Alanine aminotransferase increase was observed when the inoculum was $>10^{2}$–10^{3} HEV RNA copies/ml. Other animals such as swine and rabbits can be experimentally infected only by zoonotic genotypes. These models have been used to demonstrate the existence of extrahepatic sites of HEV replication (90–92. Human liver chimeric mice have been developed (93, 94) that can be infected with genotype 1 or zoonotic genotypes. This model may well prove a fruitful method of studying chronic infections and evaluating preclinical antiviral agents.

Studies using animal models of infection and human data have identified three distinct phases of HEV pathogenesis, namely (i) incubation period; (ii) acute hepatitis with various clinical phenotypes (see below); and (iii) convalescent phase. Cell-culture studies suggest that HEV is a noncytopathic virus and that the outcome of acute HEV infection is determined by the strength of the host's immune response. Viral replication in the liver appears about 7 days after virus transmission but the percentage of infected hepatocytes is still unclear. Viremia appears a few days before the onset of symptoms, attains its peak with that of serum alanine aminotransferase levels, continues for a few weeks, and is accompanied by prolonged fecal HEV excretion (95). Although HEV is a hepatotropic virus, immunohistochemistry experiments and detection of negative strand of HEV RNA indicate that it can also replicate in other tissues such as the gastrointestinal tract (90, 92), kidney (96), central nervous system (97), and placenta (98).

IMMUNE RESPONSES

As for many other viruses, a concerted action of innate and adaptive immunity is involved in the outcome of HEV infection and protection. However, our understanding of the

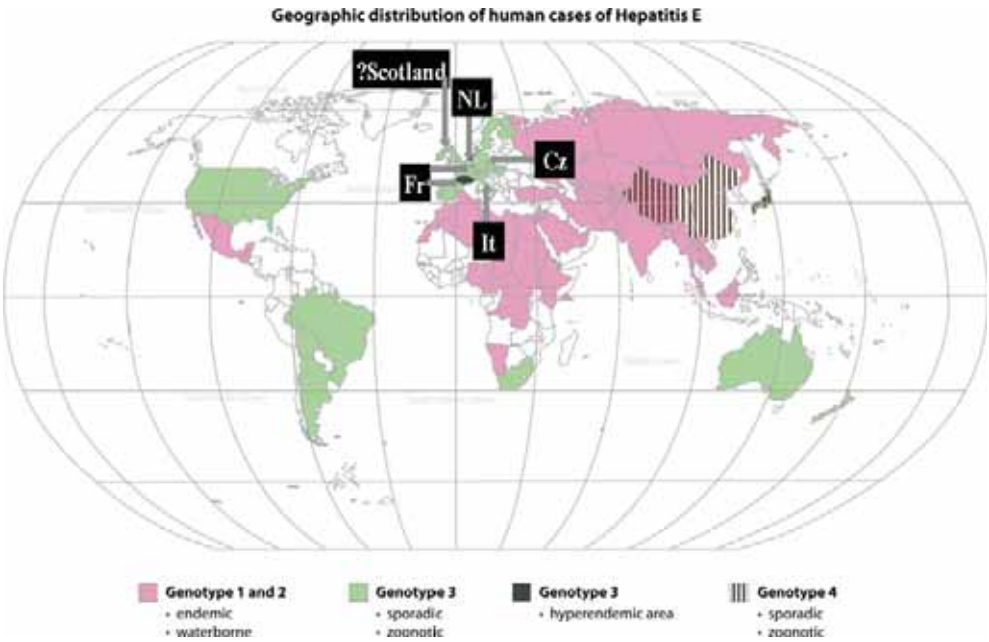

FIGURE 7. European "hot-spots" with high incidence/prevalence of HEV, include southern and northeastern France and the Netherlands (see main text). Very recent data show a huge incidence in the Czech Republic (86) and a seroprevalence in blood donors in Abruzzo, Italy of nearly 50% (216).

molecular mechanisms and the crosstalk between the host immune response pathways is still limited.

Innate Immune Responses

HEV infection elicits the production of type I and III interferon (IFN) after recognition of the viral RNA as a pathogen-associated molecular pattern (PAMP) by pattern recognition receptors (PRR). Secreted IFNs can then induce interferon-stimulated gene (ISG) transcription via the JAK/STAT signaling cascade (39, 99, 100). The ORF3 protein may enhance type I IFN production via a direct interaction with the PRR retinoic acid-inducible gene I (RIG-I). The ORF1 protein inhibits RIG-I signaling and prevents IFNβ induction by deubiquitination of RIG-I and TANK-binding kinase I (TBK1) (101, 102). In addition, the ORF3 protein has been shown to inhibit the IFN-α-induced phosphorylation of Stat 1, thus restricting its activation (103).

Natural killer cells and natural killer T cells show evidence of activation in patients with acute hepatitis E (104). Gamma-delta T cells are mobilized during acute HEV infection in transplant patients (105).

Cell-Mediated Immune Responses

Potential differences between the different genotypes related to differences in disease severity and outcome are still unknown. This is an important area of research for a better understanding of the pathogenesis of HEV infection during pregnancy. HEV-specific T-cell responses are weaker in patients with fulminant acute hepatitis E than in patients with milder disease (106). Cross-genotype specific T-cell responses are generated during acute infection and are associated with viral control (107). CD4+ and CD8+ T cells from healthy subjects previously exposed to HEV show strong and multispecific responses against HEV peptides, whereas those from immunocompromised patients with chronic infection do not (108).

Humoral Immune Response

HEV infection is associated with appearance of anti-HEV antibodies. IgM antibodies are the first to appear and persist for 6 to 9 months while IgG antibodies persists for several years. One study detected anti-HEV IgG antibodies in nearly half the patients who had hepatitis E 14 years previously (109). Anti-HEV antibodies have been shown to neutralize the virus *in vitro* (110). Importantly, this neutralization capacity is observed only with naked particles (i.e., stool derived-HEV) (14). The neutralization capacity extends to heterologous strains belonging to all the four major HEV genotypes. Current neutralization tests are based on real-time PCR, immunofluorescence foci assay, and a high-throughput assay using biotin-conjugated HEV recombinant capsid protein (111). Neutralization titers of HEV serum samples are associated with anti-HEV IgG levels. However, the anti-HEV IgG level in humans conferring absolute protection against HEV is unknown. Therefore, the duration of natural protection against HEV reinfection remains unclear.

CLINICAL MANIFESTATIONS

Acute HEV

In developing countries, hepatitis E mainly affects young adults with sporadic infections and occasional sizable outbreaks. The incubation period is approximately 40 days. The clinical phenotype ranges from asymptomatic infection, through mild self-limiting hepatitis, to acute liver failure, with an overall reported mortality of approximately 1%. Symptoms and signs of hepatitis E include anorexia, abdominal pain, nausea, vomiting, fever, general malaise, and jaundice. Biochemical evidence of hepatitis includes elevated serum levels of liver enzymes and hyperbilirubinemia, which usually return to normal within a few weeks (7).

The clinical features of hepatitis E in developed countries are similar to those seen endemically in developing nations, and are virtually indistinguishable from those observed in any form of acute viral hepatitis, except that 5% to 10% of patients present with a primarily neurological illness (see below). However, HEV infection in developed countries is commonly asymptomatic (Fig. 8) (7) and clinical illness is mostly observed in middle-aged and elderly males (72, 112).

CHRONIC HEV

The first cases of chronic HEV infection were reported in solid-organ-transplant patients (113). Among 14 patients who had been infected by HEV, 8 had persistent HEV replication for at least 6 months with increased ALT and progressive liver fibrosis (113). Thereafter, a large number of cases of chronic HEV infection have been described in transplant and other immunosuppressed patients (see below). All cases of chronic HEV infection have occurred in patients infected by genotype 3 or 4 (7). No case of chronic HEV1 or 2 infection has been reported. A study of 205 renal transplant recipients from India found no evidence of chronic infection with HEV (114).

Most data on the natural history and treatment of chronic HEV infection have been obtained in solid-organ-transplant patients. Initially, chronic HEV infection was defined as persistent replication of HEV for at least 6 months (113). However, in a large cohort of chronically infected transplant recipients, no spontaneous HEV clearance was observed between 3 and 6 months after infection without therapeutic intervention (115). Hence, it has been suggested that HEV replication for more than 3 months should be the definition of chronic infection. This definition is still a matter of debate, because recently a case of spontaneous clearance was observed 4 months after HEV infection (116, 117). In a large multicenter retrospective study that included 85 solid-organ-transplant recipients, 66% of patients developed chronic hepatitis and nearly 10% developed cirrhosis (118). Surprisingly, liver fibrosis progression was rapid and some patients developed cirrhosis only 2 to 3 years after infection (118, 119). No correlation has been observed between HEV viral load and liver fibrosis progression (120).

Chronic HEV infection and cirrhosis have been observed in both adult and pediatric solid-organ-transplant patients (121). The source and route of HEV infection in transplant recipients does not differ from that in the immunocompetent (see above). The one exception is that the grafted organ can occasionally transmit HEV infection (122) to the recipient. As in the immunocompromised patients, the majority of transplant recipients who develop HEV infection are asymptomatic (118). However, compared to immunocompetent patients, chronic HEV in immunocompromised patients usually produces mild elevations in serum transaminases (ALT typically 100-300IU/L) and HEV serology can remain negative (118). Therefore, the use of HEV PCR is mandatory in immunocompromised patients (121), both to establish the diagnosis and to monitor response to therapy.

In the first report of cases of chronic HEV infection, patients having lower CD4 and CD8 lymphocyte counts were more likely to develop chronic HEV infection,

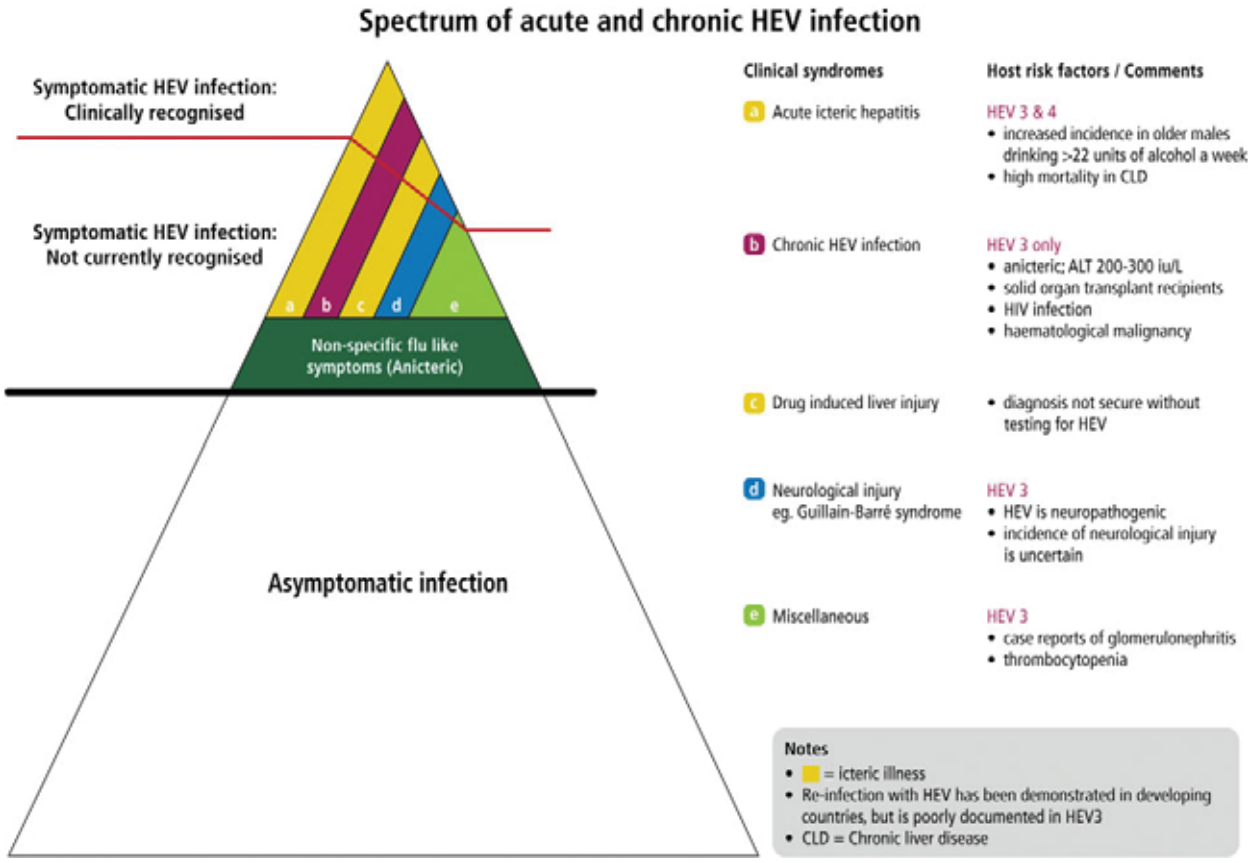

FIGURE 8. Symptomatic and asymptomatic HEV.

suggesting that more immunosuppressed patients were at higher risk of developing chronic HEV infection (113). More recently, a multicenter retrospective study analyzing risk factors for the development of chronic infection following exposure (118) suggested that liver-transplant patients had a higher risk of developing chronic infection compared to non-liver-transplant patients (118). Also, ALT and AST levels were lower at HEV diagnosis in patients who developed chronic hepatitis, suggesting a lower T-cell response in these patients, as has been demonstrated *in vitro* (31, 108, 118). In patients who developed chronic infection, the time following transplantation was shorter than in those who cleared the virus. In addition, the time since treatment of an acute rejection episode was also shorter, and patients were more often treated with tacrolimus than with cyclosporin A (118). Multivariate analysis showed that the use of tacrolimus was an independent predictive factor for the progression to chronic HEV infection (118). Large quasispecies heterogeneity was observed in patients with persistent HEV compared to those with resolving hepatitis (23, 31).

In addition to transplant recipients, chronic HEV infection and cirrhosis have also been observed in a small number of HIV positive patients, mainly in those having a low CD4 count (7, 123). HEV infection can persist in patients who improved their immune status (124). Stem-cell transplant recipients, hematological patients receiving chemotherapy (125, 126), and patients with rheumatologic or autoimmune disease receiving immunosuppressive therapy can also develop chronic HEV infection (127, 128).

COMPLICATIONS OF HEV

Hepatic Complications

The mortality of hepatitis E in developing countries is approximately 1%, due largely to subacute hepatic failure in patients with preexisting chronic liver disease (12-month mortality up to 70%) (53). Fulminant hepatic failure occurs in pregnant women in the third trimester (mortality approximately 20%) (129). The latter is a unique characteristic of hepatitis E, and historical studies of outbreaks of hepatitis with deaths in pregnant females show that waterborne hepatitis E was probably quite common in Continental Europe in the 19th century (5). Deaths occur toward the end of pregnancy and are mainly caused by a combination of fulminant hepatic failure associated with obstetric complications, including bleeding and eclampsia. In addition, the unborn child frequently does not survive (129, 130). Infected women have higher viral loads than nonpregnant women (131, 132), and several studies have shown differences in immunological and hormonal responses in pregnant women with fulminant hepatic failure caused by hepatitis E (133). However, the cause of high maternal mortality in patients with hepatitis E remains uncertain.

In developed countries, the mortality is unknown. Hospital-based case series report a mortality ranging from 3% to 10% (72, 134). Deaths mostly occur from acute liver failure in older men with preexisting chronic liver disease. Occasionally fulminant hepatic failure occurs in patients without underlying chronic liver disease (135). As noted

above, transplant recipients can develop rapidly progressive cirrhosis and liver failure. This required liver transplantation in some cases, and a number of patients died.

Extrahepatic Complications

An increasing number of syndromes affecting organ systems other than the liver have been reported that are temporally associated with hepatitis E. These occur during both acute and chronic infection and in developing and developed countries (Table 1). For most of these extrahepatic manifestations, a causal relationship has yet to be established. However, it is likely that in the case of renal and neurological syndromes that the relationship with HEV infection is causal.

Renal

Renal impairment in association with HEV has been defined best in immunosuppressed transplant recipients with chronic infection. Renal function deteriorates during the course of infection and improves with viral clearance. Renal biopsy shows a range of renal injury, including membranoproliferative and membranous glomerulonephritis and relapses of IgA nephropathy with or without cryoglobulins (136, 137). Renal injury has also been reported in an immunocompetent patient with acute HEV infection who developed crescentic glomerulonephritis with cryoglobulins that improved after viral clearance (138).

Neurologic

Approximately 100 patients with a range of neurologic damage temporally related to hepatitis E infection (139) have been reported to date (Table 1), and there are many more cases not yet published. Neurologic injury was described in 5% to 8% of patients from two large cohorts of patients with mainly acute and chronic hepatitis E from Toulouse, France and Cornwall, England (134, 140). A range of neurologic pathology was documented in this study including Guillain-Barré syndrome, neuralgic amyotrophy, inflammatory polyradiculopathy, ataxia/encephalitis, and peripheral neuropathy. In four patients with chronic HEV infection, HEV RNA was observed in the cerebrospinal fluid. The incidence of neurological injury is underrecognized because most patients present primarily with a neurologic illness without jaundice. The liver function tests are usually only mildly abnormal and in a minority of patients were completely normal at the time of presentation.

Case-control studies of hepatitis E in Guillain-Barré syndrome (GBS) from the Netherlands (141), Bangladesh (142), and Japan (143) all illustrate that patients with GBS are significantly more likely than controls to have evidence of HEV infection. Between 5% to 10% of GBS patients have evidence of infection at the start of their illness, and interestingly, in a minority viral RNA was found in serum and stool but not CSF.

Neurologic amyotrophy (NA) is a postinfectious immune-mediated neuropathy characterized by damage to the brachial plexus, resulting in severe shoulder pain, muscular atrophy, weakness, and sensory disturbance (144). An Anglo/Dutch cohort study of patients with NA showed that 10% had evidence of HEV infection at presentation (145). Patients with HEV-associated NA have been reported only with HEV3 and are mainly middle-aged males who typically have bilateral neurologic symptoms and signs in the upper limbs. This contrasts to NA without HEV infection where the neurologic damage is unilateral affecting the dominant arm. Central nervous system involvement has also been seen in 12 cases of both acute and chronic infection, and in some cases the outcome was poor, with several deaths (139).

The mechanisms whereby HEV causes neurologic injury are unknown. It could be immune-mediated, or HEV might be directly neurotropic. Some evidence suggests that HEV is neurotropic, and in some cases HEV RNA has been recovered from the CSF (139). The HEV quasispecies in the CSF was different than that recovered from the serum in one chronically infected patient (146). Another patient with a peripheral neuropathy and chronic HEV (HEV RNA in both serum and CSF) showed resolution of his neurological symptoms, which was temporally related to viral clearance with ribavirin and interferon (147). Recent *in vitro* and *in vivo* evidence shows that HEV can grow well

TABLE 1 Extrahepatic manifestations of acute and chronic hepatitis E

System	Feature	Comments
Neurologic	• Guillain-Barré syndrome	• >40 cases reported worldwide
	• Neuralgic amyotrophy	• Acute HEV 3 only, middle-aged males
	• Meningoencephalitis	• Acute and chronic HEV; prognosis poor
	• Mononeuritis multiplex	• 6 cases reported, HEV3
	• Myositis	• 2 cases reported, raised creatine kinase
	• Bell's palsy, vestibular neuritis, and peripheral neuropathy	• Case reports only
Renal	• Glomerulonephritis and IgA nephropathy	• Mainly described in chronic infection with HEV3 but also seen in immunocompetent patients with acute infection
Hematological	• Thrombocytopenia	• Mild thrombocytopenia is common, occasionally severe
	• Monoclonal immunoglobulin	• Reported in 25% of cases of acute HEV, clinical significance uncertain
	• Cryoglobulinemia	• Occurs mainly in association with renal disease
	• Aplastic anemia	• Case reports only
Other	• Acute pancreatitis	• 55 cases worldwide, HEV1 only
	• Arthritis, myocarditis, and autoimmune thyroiditis	• Single case reports only

There is good evidence to support a causal role for HEV in Guillain-Barré syndrome, neuralgic amyotrophy, meningoencephalitis, and renal syndromes. For the other extrahepatic manifestations, causality remains to be established.

on a range of neurological cell lines and can cross the blood brain barrier in animals (97, 148).

LABORATORY DIAGNOSIS

HEV infections can be diagnosed either indirectly by detecting anti-HEV antibodies in the serum or directly by detecting HEV RNA or capsid antigen in the blood or other body fluids (121, 149). Current data indicate similar performance for all HEV genotypes and subtypes. An initial incubation period of 2 to 6 weeks (Fig. 9) usually precedes the IgM response, which is detected around the time the alanine aminotransferase activity increases, and persists for 6 to 9 months (150). The IgG response can be delayed; it persists for several years, although the exact duration of this response remains uncertain. HEV RNA becomes detectable in the blood and stools during the incubation period and persists for around 4 weeks (blood) and 6 weeks (feces). Capsid antigen persists in the blood for about the same time (151).

Serology

The antigens used in enzyme immunoassays (EIA) are usually recombinant ORF2 and/or ORF3 proteins from an HEV1 strain. Sera from patients infected with other genotypes generally cross-react adequately due to shared ORF2/ORF3 epitopes. The diagnostic performance of IgM and IgG assays varies considerably and must be evaluated carefully (152).

The presence of anti-HEV IgM in the serum is a key marker of an acute infection. Both conventional microplate and rapid immunochromatographic commercial assays have been developed. A validated PCR assay was used as a reference in studies that showed that the sensitivity of these assays was >97% for immunocompetent patients and 80–85% for immunocompromised patients; their specificity was >99.5% 153–155.

The presence of anti-HEV IgG alone is a marker of past infection. The limits of detection of commercial anti-HEV IgG assays vary from 0.25 WHO unit/ml to 2.5 WHO unit/ml (WHO reference reagent established in 2002; National Institute for Biological Standards and Control Code 95/584). A specific, sensitive assay (detection limit: 0.25 WHO unit/ml) has been used to obtain a clearer picture of HEV epidemiology (74). This assay generally produces higher estimates of anti-HEV IgG seroprevalence, as highlighted in a meta-analysis of studies of HEV prevalence in Europe (156). Nevertheless, the high clinical sensitivity does not reflect poor specificity; adult populations in Fiji, New Zealand, and Scotland who have a very low seroprevalence (<5%) were identified using this assay. Similarly, the seroprevalence is only 2% in children aged 2 to 4 years living in southwest France despite the fact that this is a hyperendemic area (157). Knowing the anti-HEV IgG concentration could be useful for estimating the risk of reinfection after a natural infection or vaccination in clinical trials. One study demonstrated that HEV immunocompromised patients with low anti-HEV IgG concentration (<7 WHO unit/ml) could become reinfected and that this could become chronic (55).

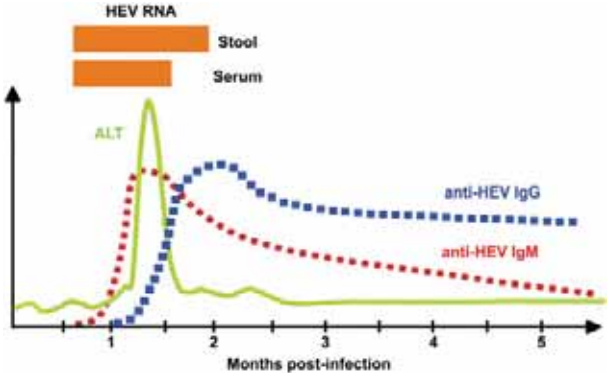

FIGURE 9. Kinetics of hepatitis E virus markers during an acute infection.

Viral RNA Detection

HEV RNA can be detected and quantified in the blood, stools, and other body compartments using nucleic acid amplification technologies with primers targeting regions that are conserved among HEV genotypes. Most real-time PCR assays, including commercial assays, target ORF3 (158, 159). A transcription-mediated amplification assay (Procleix HEV) performed on a fully automated platform (Panther system) is well adapted for high-throughput testing (160). Lastly, the newly developed loop-mediated isothermal amplification (LAMP) assay provides a one-step, single-tube amplification of HEV RNA without special equipment (161).

The assays based on nucleic acid amplification all require the WHO international reference panel for HEV RNA genotypes for their validation and comparison of their performance. The limit of detection of current assays is 7 to 80 IU/ml. This feature is relevant not only for diagnosis but also for defining an optimal strategy (individual or minipool testing) for blood screening.

HEV RNA can be characterized by sequencing different regions of the HEV genome such as ORF2 or OFR1 polymerase. This can then be used to determine the HEV genotype/subgenotype and hence to identify the source of infection and the mutations in the polymerase of virus infecting patients whose ribavirin therapy fails (162, 163). Current studies are examining the prognostic value of these mutations.

Antigen Detection

HEV infections can be diagnosed by detecting the HEV capsid antigen using a commercial sandwich EIA. One study found that the specificity was excellent and the lowest HEV RNA concentration detected was 800 to 80,000 IU/ml using serial dilutions with HEV RNA-negative anti-HEV antibody-negative plasma (164). The diagnostic sensitivity for an acute HEV infection was 91%, with no significant difference between immunocompetent (88%) and immunocompromised (94%) patients (164). Although HEV RNA testing is the gold standard for detecting an active infection, testing for HEV capsid antigen is technically simpler, less expensive, and faster. Therefore, this could be a valuable alternative for diagnosing HEV infections for laboratories with no molecular diagnostic facilities.

Diagnostic Algorithm

The good performance of anti-HEV IgM assays and their wide availability makes them suitable for use as a first-line diagnostic assay (Fig. 10). Immunocompromised patients should be tested for HEV RNA in three clinical settings: (i) if the anti-HEV IgM is negative and ALT activity is elevated, because the immune response of these patients is frequently

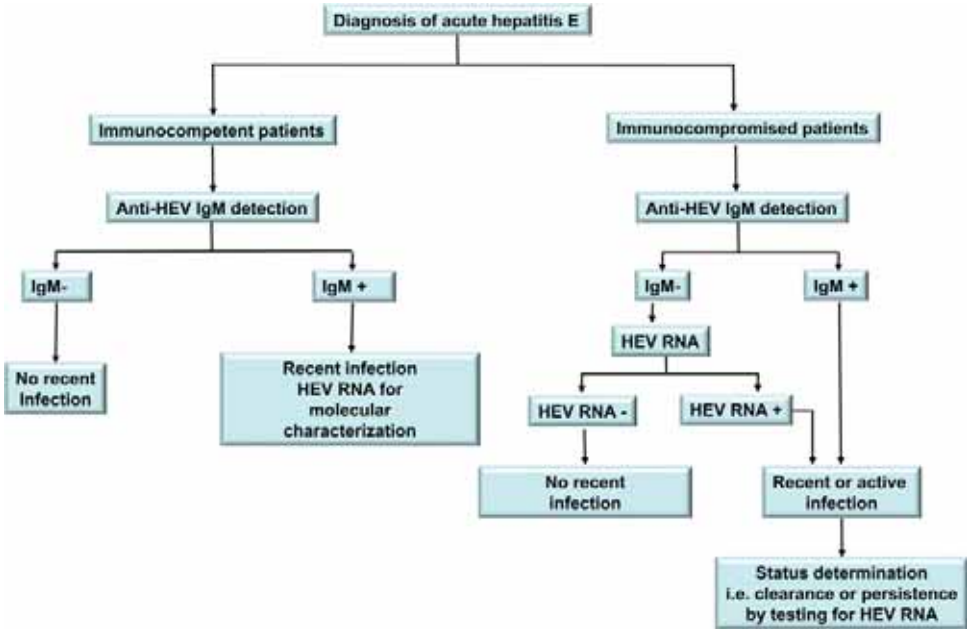

FIGURE 10. Flow diagram for diagnosis of acute hepatitis E virus infection.

impaired, (ii) to identify a chronic infection if the HEV RNA persists for 3 to 6 months, and (iii) to monitor HEV RNA so as to manage a chronic infection after immunosuppression has been reduced or after starting antiviral therapy. Ribavirin therapy is used to reduce the HEV RNA concentration in the blood to undetectable levels. A negative HEV RNA concentration in the feces could also be necessary to indicate successful eradication of the virus after treatment has been interrupted (165).

TREATMENT

Acute Infection in the Immunocompetent

Most patients with hepatitis E require no treatment because they have a mild self-limiting illness. Patients with severe infections, including patients with fulminant hepatitis, should receive appropriate supportive care including, where necessary, admission to an intensive care unit experienced in dealing with liver disease (see Hepatitis chapter). A few patients who have presented with severe acute HEV infection, including patients with preexisting chronic liver disease, have been given ribavirin (166, 167). A rapid decrease in HEV RNA concentration occurred with clearance of HEV. However, there are no placebo-controlled data available either in this setting or in patients with chronic infection.

Chronic Infection in Solid-Organ-Transplant Patients

Clinical studies have shown that tacrolimus is a more potent immunosuppressive drug than cyclosporin A (168). *In vitro*, both calcineurin inhibitors (tacrolimus and cyclosporin A) and mammalian target of rapamycin inhibitors (sirolimus and everolimus) promote HEV replication (169, 170). Conversely, mycophenolic acid, an inosine monophosphate dehydrogenase inhibitor, inhibits HEV replication (169).

The treatments for HEV infection were initially evaluated in solid-organ-transplant patients with chronic HEV infection. In this setting reduction of immunosuppression that targets T-cells prompts viral clearance in up to 30% of patients (118). Patients who clear the virus by reducing immunosuppressive therapy achieved a lower tacrolimus trough level and needed lower daily doses of steroids compared to those who remained viremic (119). Hence, a reduction in immunosuppressive therapy seems to be the first-line therapeutic option. However, since two-thirds of patients fail to clear the virus despite the reduction of immunosuppressive therapy and since this maneuver cannot be safely performed in high-risk immunological patients, antiviral therapy has been employed.

Interferon has a moderate antiviral activity *in vitro* (170, 171) and has been given to a few liver-transplant patients, mostly for a 3-month period. A sustained virological response was obtained in all patients (172–174). However, the use of interferon is problematic in transplant patients because it increases the risk of acute rejection due to its immunostimulatory effect (175). This prompted several groups to use ribavirin alone in solid organ transplant recipients with chronic hepatitis. Unexpectedly, the results have been impressive (176–178). In a retrospective French study, the effect of ribavirin alone was assessed in 59 solid-organ-transplant recipients with chronic HEV infection (179). The majority of patients received ribavirin at the daily dose of 600 mg (n=17) or 800 mg (n=17) for range of 1 to 18 months; most patients (n=36) were treated for 3 months. Anemia was the main side effect and required ribavirin dose reduction in one-third of the patients, temporary cessation of therapy in two patients, and the use of recombinant erythropoietin in up to 40% of the patients (179). The sustained viral response (SVR) at 6 months after cessation of therapy was 78% (n=46) (179). Two patients were lost to follow-up, another patient was initially a nonresponder, and 10 other patients relapsed after ribavirin therapy was stopped. The nonresponder was retreated and achieved SVR. Six of the 10 relapsers who were initially treated for 3 months were retreated for 6 months. SVR was then observed in 4 of these patients; the 2 remaining patients also had viral clearance but

with limited follow up. Hence, the overall SVR was 85% (179). A lower lymphocyte count at the initiation of ribavirin therapy and persistent HEV shedding in the stools at the end of therapy (165) have been associated with more relapses after ribavirin is stopped (179). A decrease in HEV RNA concentration of more than 0.5 log copies/mL within the first week after initiating ribavirin therapy was found to be a predictive factor for SVR (180). However, ribavirin trough levels early or late after the initiation of therapy did not affect virological response or SVR (180).

Ribavirin monotherapy has become the treatment of choice for chronic HEV infection, but the mechanism of action remains unclear. It has been suggested that ribavirin inhibits HEV replication through depletion of guanosine triphosphate (GTP) (181). *In vitro*, the combination of ribavirin and mycophenolic acid has a synergistic anti-HEV effect (169). *In vivo*, no difference in the slope of HEV RNA concentration and the number of SVRs was observed between transplant recipients who received ribavirin either with or without mycophenolic acid (180). It has been recently shown that ribavirin increases viral heterogeneity and that ribavirin-induced mutagenesis seems to be reversible after therapy is stopped (182).

Cases of ribavirin treatment failure have been reported in patients who have the *G1634R* mutation in viral polymerase (183). This mutation does not provide ribavirin resistance *in vitro* but increases the replicative capacity of HEV (183). In a series of 63 transplant recipients with chronic infection, 23 (36.5%) had detectable *G1634R* variants before therapy (163), but the presence of the *G1634R* mutation before treatment did not have any impact on the early virological response, on the SVR, or on virological response after retreatment (163). Interestingly, *G1634R* variants were detected during therapy in patients who relapsed after ribavirin cessation (182). Other dominant mutations were described in the polymerase region (i.e., *K1383N*, *Y1587F*, *D1384G*, *V1479I*, and *K1398R*) (171). On the one hand, these mutations increase the replicative capacity of HEV, and on the other they improve the antiviral activity of ribavirin (162).

In cases of ribavirin treatment failure there is currently no alternative effective therapy of proven value. Sofosbuvir may inhibit HEV replication *in vitro* and the anti-viral effect is increased when combined with ribavirin (184). However, it failed to decrease HEV replication (185) in one patient chronically infected with both HEV and HCV.

Chronic Infection in Non-Solid-Organ-Transplant Patients

A few case reports and case series have been published on the treatment of hematological malignancy patients (i.e., patients receiving chemotherapy or stem-cell-transplant recipients). Similar to solid-organ-transplant recipients, hematology patients have been successfully treated with pegylated interferon or ribavirin (186–188). Pegylated interferon, ribavirin, or both were also successfully used in HIV patients (147, 189–191).

Extrahepatic Manifestations

Antiviral therapy has been used in patients presenting HEV-induced neurological symptoms such as Guillain-Barré syndrome (140). Although viral clearance is achieved, the effects on the outcome of neurological symptoms is uncertain. In contrast, antiviral HEV therapy appears to be very effective in treating patients who developed HEV-associated kidney complications (136, 138).

PREVENTION

General Risk Reduction

Providing access to clean drinking water is the most important approach to preventing hepatitis E in developing countries, but this can prove problematic when the sanitary infrastructure fails. For example, recent outbreaks have occurred in African refugee camps. Prevention of locally acquired zoonotic infection with HEV in developed countries is more difficult, because there are a number of animal hosts and several possible routes of infection (Fig. 4). Prevention strategies include thorough cooking of pork-containing foods, appropriate care and precautions when handling uncooked pork, and screening of blood donors to prevent transfusion-transmitted cases.

Vaccination

HEV 239 vaccine (Hecolin®, Xiamen Innovax, China) consists of pORF2 amino acids 368 to 607 expressed in an *E. coli* system that can self-assemble *in vitro* into virus-like particles with a diameter of 20–30 nm. It has been tested in both animals and humans. Two doses of 5 μg, 10 μg, or 20 μg HEV 239 adsorbed in alum adjuvant when administered 4 weeks apart to monkeys induce comparable antibody responses that protect the animals from HEV1 and HEV4 (192). In humans, Phase 1, 2, and 3 clinical trials have been reported from China (193–195). In these studies, the vaccine formulation contained recombinant particulate HEV 239 antigens adsorbed to 0.8 mg of aluminum hydroxide and suspended in 0.5 ml of buffered saline and was given by intramuscular injection.

HEV 239 vaccine appears to be effective at preventing HEV infection. In the Phase 3 clinical trial, over 100,000 participants were randomly assigned to receive three doses of 30 μg HEV 239 vaccine or the hepatitis B vaccine at months 0, 1, and 6 (195). During follow-up for 12 months after the last dose, no HEV 239 vaccine recipients contracted hepatitis E, but 15 cases of hepatitis E occurred in the control group, indicating a vaccine efficacy of 100% (95% CI, 72–100). In participants who received at least one dose the efficacy was 96% (95% CI, 66–99); and those receiving two doses and before the third dose the efficacy was 100% (CI 9–100). In an extended efficacy study (up to 4.5 years after vaccination), the efficacy of HEV 239 was 93% (95% CI, 79–98) (196). Of the 29 identified hepatitis E cases whose viral isolates were sequenced, 26 were infected with HEV4 (3 in the vaccine group and 23 in the control group), and 3 with HEV1 (1 in the vaccine group and 2 in the control group). Data from the Phase 2 clinical trial shows that 2 doses of 20 μg HEV 239 (administered at months 0 and 6 or months 0 and 1) effectively protect from HEV infection with an efficacy of 85% (95% CI, 10–99) for at least 6 months (194).

HEV239 vaccine is well tolerated and no serious adverse events have been reported. In the Phase 3 clinical trial local adverse events (pain and swelling around the injection site) occurred more frequently in the HEV 239 group than in the control group (195). During extended follow-up, similar numbers of participants in both the vaccine and placebo groups experienced serious adverse events, none of which were related to vaccination (196). Thirty-seven pregnant women in the HEV 239 vaccine group and 31 pregnant women in the control group were inadvertently vaccinated. The vaccine was well tolerated in the pregnant women, with only one woman reporting mild inoculation-site pain. The

weights, body lengths, and gestational ages of the newborns were comparable in both groups (197).

HEV 239 vaccine was licensed for use in China in 2012 (193) but is currently not available in other countries. It may be potentially of utility in an outbreak setting in developing countries, because its efficacy after two doses 1 month apart is 100% (194). However, the issue of vaccine availability in developing countries in need is complex. There are a number of issues that have impeded the use of HEV239 in countries other than China, including lack of vaccine prequalification and insufficient data regarding efficacy against HEV1, safety and efficacy in pregnant women, and efficacy against genotype 1. In both developing and developed countries, hepatitis E has a poor prognosis in patients with chronic liver disease. Such individuals could be considered for vaccination against HEV. However, the safety and efficacy of HEV239 in patients with chronic liver disease is currently unknown. HEV239 is safe and effective in carriers of HBsAg (198), but how many of the individuals in this study were cirrhotic is unknown. In developed countries, a safe and effective vaccine could potentially be used to prevent chronic infection in the immunosuppressed. The safety and efficacy of the HEV239 in this group of patients is currently unknown. In developed countries with high levels of circulating HEV, there has been interest in vaccination of farmed pigs as a method of reducing exposure of humans. The acceptability of this approach to the farming community is likely to be problematic, as pigs infected with HEV are asymptomatic. In addition, its efficacy in terms of impact on human infection is uncertain, because HEV is ubiquitous in the environment and there are several other animal hosts.

A number of other approaches to vaccine development are ongoing, including a DNA vaccine, chimeric virus particles, fusion proteins, and an oral vaccine (199–209). None of these novel vaccine candidates have yet been used in humans.

HEV and the Blood Supply

Because HEV infections are widespread and blood donors are often asymptomatic, there is a risk of virus-containing blood donations. Transfusion-related HEV infections have been documented in several countries, including the United Kingdom, France, Germany, and Japan (121). The blood components involved in HEV transmissions were fresh frozen plasma (solvent/detergent-treated, amotosalem-treated, secured by quarantine), pooled or apheresis platelets, and red blood cells. In a retrospective study in England of 43 recipients given HEV-contaminated blood products and followed up, the transmission rate was 42%, but only 1 patient developed symptomatic hepatitis. The patients who were given blood products with high HEV RNA concentrations were more likely to become infected (71). A French kidney transplant patient was infected with HEV when treated by plasma exchange for acute humoral rejection (210).

Published data indicate that the minimum infectious dose in donations is 3.85 log HEV RNA IU (211). This information is crucial for assessing the capacity of HEV inactivation/removal during manufacturing steps of plasma-derived medicinal products and the accompanying risk of infection. The same sensitive validated assay was used in HEV seroprevalence studies of blood donors, identifying three levels of endemic virus frequency in developed countries, low (<10%), intermediate (10%–20%), and high (>20%) (212). The incidence of HEV viremia was 1:600 to 1:2600 in countries where the virus was highly endemic, including France, Germany, The Netherlands, Japan, and China, and 1:2800 to 1:14500 in the other two categories, including the United Kingdom, United States, Australia, and New Zealand. However, HEV seroprevalence and viremia may vary from one region to another within a country, as illustrated by French studies (69, 213, 214). These epidemiological data are essential for defining optimal policies for screening blood donations for HEV. Universal screening of blood donors for HEV started in Ireland in 2016. Targeted screening of donors in the United Kingdom also started in 2016, with screening of donors whose blood products are destined for high-risk groups, including the immunosuppressed (215).

CONCLUSIONS

Our understanding of HEV has changed completely in the last 10 years. It was previously considered to be a waterborne pathogen affecting a few resource-poor settings. We now know that it is a virus of global significance and is also endemic in many resource-rich countries where it is largely locally acquired, anthropozoonotic, and causes both acute and chronic infection. Because it is a very common infection in such settings, and commonly asymptomatic, it has found its way into the human blood supply, and viremic donors are surprisingly frequent in many countries in Europe. Whether and how donors should be screened for HEV remains a "hot topic" in the blood transfusion community. The clinical phenotypes of hepatitis E in humans are still emerging. It is now clear that HEV can cause a number of extrahepatic manifestations and in particular a range of neurological injuries. The range, frequency, and mechanisms of HEV-associated neurological injury are as yet uncertain. The global burden of HEV is unknown. The most recent figure is an underestimate, because it utilized data from 2005 from a limited number of developing countries and took no account of the paradigm shift implicit in the current thinking of HEV as a global pathogen. Maybe in another 10 years we will have a much clearer idea about many of these aspects of HEV, which is proving to be one of the more successful zoonotic viruses currently affecting humans.

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Astrovirus

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Astroviruses are present in a wide variety of animal species, including mammals and birds. In many species, including humans, these viruses are associated with gastrointestinal diseases and, more recently, with encephalitis and diverse neurological manifestations. Human astroviruses (HAstVs) were first identified in fecal samples of children with diarrhea by electron microscopy as small particles with a star-like morphology, the feature that Madeley and Cosgrove (1) used in 1975 to name this group of viruses (*astron*-star in Greek). This morphology, however, is observed in only a small proportion of the particles present in stool samples, and expertise in electron microscopy is required for their identification. Development of more sensitive and specific diagnostic methods, such as enzyme immunoassays (EIA) and reverse transcription, coupled with polymerase chain reaction (RT-PCR), in diverse formats have revealed that HAstVs represent serious gastrointestinal pathogens that affect distinct groups in the population. Adaptation of HAstVs to tissue culture and the molecular characterization of human, as well as of animal, viruses have contributed to recent advances in the understanding of their molecular biology; however, an animal model to study HAstV pathogenesis is still required.

The *Astroviridae* family consists of icosahedral nonenveloped viruses with characteristic features that distinguish them from other naked viruses with a monopartite RNA genome, like those in the *Caliciviridae* and *Picornaviridae* families. Members of the family *Astroviridae* contain a single-stranded RNA (ssRNA) genome of positive polarity organized in three open reading frames. The virions are formed by proteins that result from the proteolytic processing of a precursor polypeptide (2, 3). During infection with astroviruses, a viral subgenomic RNA (sgRNA) is used as template for the synthesis of the structural proteins (4). Additional features used to distinguish this family are the lack of RNA-helicase and methyltransferase domains in the nonstructural proteins and the use of a ribosomal frameshifting mechanism to translate the viral RNA-dependent RNA polymerase (RdRp) (5).

VIROLOGY

Classification

Astroviruses are grouped into the family *Astroviridae*, which is divided into two genera: *Mamastrovirus*, including viruses infecting mammals (humans, lambs, calves, deer, piglets, kittens, mice, dogs, bats, rabbits, rats, sea lions, dolphins and mink among others), and *Avastrovirus*, including viruses that infect avian species (turkeys, chickens, ducks, pigeons and guinea fowl among others). Based on their reactivity in antibody neutralization assays, HAstVs were initially classified into eight serotypes (HAstV-1 to HAstV-8) (6–8). Since the sequence of the hypervariable carboxy terminus of the structural protein correlates with these serotypes, they are now commonly inferred by sequencing DNA fragments obtained by RT-PCR. Next-generation sequencing and metagenomic studies have recently detected genetically diverse astrovirus strains, and the astrovirus taxonomy is now based on the degree of identity of the amino acid sequence of the complete capsid polyprotein (9). The first novel HAstV was reported in 2008 by deep sequencing of a sample isolated from a child with acute diarrhea (10). Based on the most recent classification, at least four genotypes of astrovirus that infect humans are now recognized. The classical 8 original serotypes are classified now as *Mamastrovirus* genotype 1, and the novel HAstVs that include the MLB, VA and HMO virus lineages are classified into three additional genotypes (9, 11). A fifth genotype of HAstV has been recently proposed to exist (12). Of interest, the novel HAstVs are more closely related to animal astroviruses. In total, 33 mammalian and 11 avian different genotype species have been described to date (11).

Composition of the Viruses

Astroviruses are 28 to 30 nanometer particles with small projections from the surface and occasional star-like structure observed by electron microscopy of fecal samples and infected cell cultures (1) (Figs. 1A and 1B). Recently, the three-dimensional structures of immature (see below) and a proteolytically processed, fully mature infectious HAstV were obtained by cryoelectron microscopy at approximately 25Å-resolution (13) (Fig. 1C). The structure of both types of particles showed a solid capsid shell with a diameter of approximately 350Å and a T=3 icosahedral symmetry. The uncleaved, immature HAstV-8 particles display 90 dimeric spikes, compared to 30 globular spikes on the cleaved, mature and fully infectious HAstV-1 particles, located on the icosahedral 2-fold axes. Both immature and mature particles display the same diameters (440 Å). One hundred and eighty copies of either a 70- or a 90-kilodalton protein are

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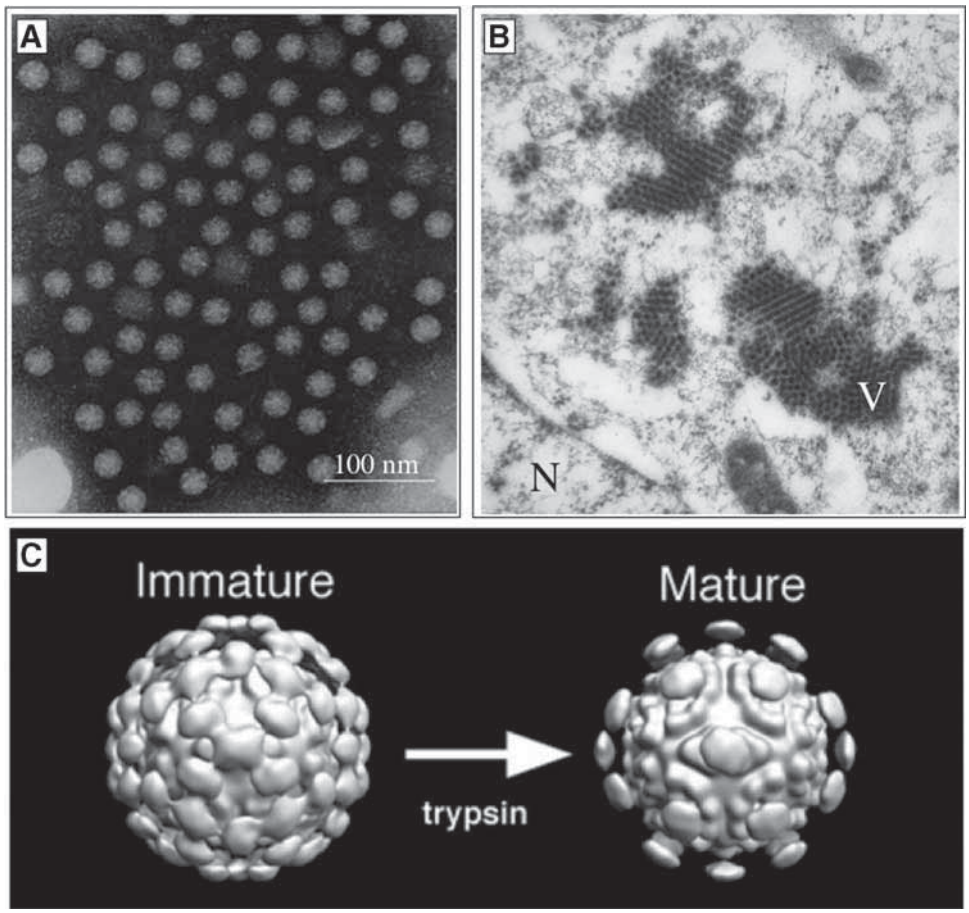

FIGURE 1 (A) The six- and five-point star-like morphology of astrovirus can be observed in fecal samples by negative staining and EM. (Reprinted from reference 1 with permission.) (B) Paracrystalline arrays of human astrovirus particles observed by transmission EM in infected Caco-2 cells; virus clusters (V) are usually localized at the periphery of nuclei (N). (C) Three-dimensional electron cryomicroscopy density maps of immature and mature HAstV. Mature virions only display 30 of 90 spikes after proteolytic cleavage. (Reprinted from reference 13 with permission.)

predicted to form the virions (13). The crystal structure of the dimeric surface spike of HAstV-1 and turkey astrovirus 2 (TAstV-2) have been also determined, and putative receptor binding sites with amino acid compositions characteristic for polysaccharide recognition (14, 15). Surprisingly, the overall TAstV-2 capsid spike showed only a distant structural similarity to the human astrovirus spike (15).

The astrovirus genome varies in length, depending on the species of origin, ranging from 6.12 kilobases (HAstV MLB-3) to 7.72 kilobases (duck astrovirus 1) (16, 17). The genome of classical HAstVs is around 6.8 kilobases; it is polyadenylated at its 3'-end, and, at its 5'-end, has covalently attached a viral protein genome-linked (VPg) (18, 19). The genome contains three open reading frames (ORFs) (Fig. 2), each encoding a polyprotein that is proteolytically processed into smaller products during the virus replication cycle. The two ORFs located towards the 5'-end of the genome, designated ORF1a and ORF1b, encode nonstructural proteins that are involved in replication and transcription of the genomic RNA, as well as in other functions relevant for virus replication, like the proteolytic processing of the precursor viral polypeptides (5). The ORF2, localized at the 3'-end, encodes the structural proteins (3, 20) (Fig. 3). As a positive-sense ssRNA virus, the RNA extracted from virions is able to initiate a productive infection (18, 21). Infectious RNA has also been transcribed from a full-length cDNA clone derived from the genome of a HAstV-1 strain (22).

The protein product of ORF1a, called nsp1a, is approximately 920 amino acid residues in length. Nsp1a is predicted to have a serine protease motif and a VPg protein that shows similarity to the VPg protein of calicivirus (18, 19). The VPg protein is covalently linked to its RNA 5'-end and is necessary for virus infectivity. One specific region of nsp1a downstream from the VPg, in which insertion/deletions are found, has been suggested to have a role in RNA replication, since strains with differences in that region synthesize distinct amounts of viral RNA (23) (Fig. 2). In addition, a phosphorylated protein derived from the carboxy-terminal end of nsp1a has also been implicated in RNA replication (24, 25). The ORF1b encodes the viral RdRp of 515 to 528 amino acids (26).

Proteins nsp1a and nsp1ab are processed at their amino terminus by a cellular protease, probably a signalase, to release a 20-kilodalton protein (27, 28) that has motifs in common with the pestivirus RNA helicase (9, 29), although the helicase activity of this protein has not been detected.

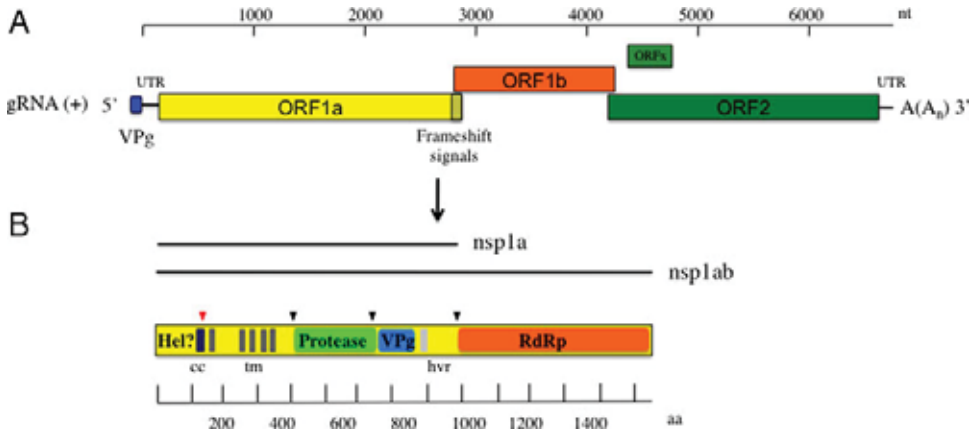

FIGURE 2 Astrovirus genome organization and synthesis of the nonstructural proteins. (A) The genome of astrovirus (shown here for HAstV-8) is organized into three ORFs: 1a, 1b, and 2. A fourth hypothetical ORFx is shown. The genomic RNA is a positive-sense RNA of around 6.8 kilobases that has a VPg protein covalently bound to its 5'-end and a polyA tail at the 3'-end. (B) Three nonstructural proteins have been positively identified: the viral protease, a VPg protein, and the RNA-dependent RNA polymerase (RdRp). In addition, other conserved structural motifs in the nsp1a polyprotein have also been identified: a putative helicase domain (Hel), a coiled-coil (cc) domain, several transmembrane domains (tm), and a hypervariable region (hvr). The RdRp is synthesized as part of a long polyprotein called nsp1ab, that results from translation of ORF1a and ORF1b as a single polypeptide, through a ribosomal frame shift mechanism directed by highly conserved cis-acting sequences (the heptanucleotide AAAAAAC and one pseudoknot) localized in the overlap region between ORFs 1a and 1b (26). The intermediate protease (viral and cellular) cleavage products of polyproteins nsp1a and nsp1b, as well as the final produced polypeptides, are not shown here, but can be consulted for HAstV-8 in other publications (28, 40). The cellular protease (red arrow head) and viral protease (black arrow heads) cleavage sites are indicated.

Most of the downstream cleavage events are thought to be carried out by the viral serine protease. Proposed sites for processing have been mapped in HAstV-1 nsp1a, Val409-Ala410, and Glu654-Ile655 to yield the viral protease (30) and in Gln664-Lys665 and Gln755-Ala756 to generate the VPg protein (18) (Fig. 2).

The structural proteins encoded in ORF2 are translated from a subgenomic RNA (sgRNA) of approximately 2.4 kilobases as a polyprotein precursor of 87 to 90 kilodalton (Fig. 3). Two domains can be clearly distinguished in this protein; the first 415 amino acid residues form the highly conserved amino-terminal domain (more than 80% identical among classical HAstVs) with two small variable regions, while the second domain includes the hypervariable region downstream of amino acid residue 416, whose sequence identity among different HAstVs can be as low as 36% (31, 32). The hypervariable domain forms the spikes of the particles and has been proposed to be involved in the first interactions of the virus with the host cell (14, 33) (Fig. 3).

The ORF2 primary translation product is processed to yield three final proteins that are present in fully infectious virions. Thus, the intracellular particles of HAstVs are constituted by polyprotein VP90, while extracellular virions are formed by either VP70 or its final cleavage products, VP34, VP27, and VP25. Proteins in the size ranges 32 to 35, 26 to 29, and 24 to 26 kilodaltons have been found as final cleavage products of the structural polyprotein of different HAstV strains, suggesting that trypsin processing of the precursor capsid polypeptide is similar in other HAstVs (2, 20, 34). Neutralizing antibodies recognize epitopes on the 26 and 29 kilodalton proteins, which are derived from the hypervariable region of the precursor polypeptide (20).

Biology

HAstVs were originally isolated in primary human embryonic kidney (HEK) cells and subsequently adapted to grow in a continuous monkey kidney epithelial cell line (LLCMK2), although these cells could not be infected directly with astrovirus extracted from fecal specimens. However, HAstVs can grow in a wide variety of cell lines, mainly of human and monkey origin, although with different efficiencies (35). Human cell lines of intestinal (Caco-2, T84, and HT-29) and hepatic (PLC/PRF/5) origin efficiently support the growth of different HAstV serotypes. Baby hamster kidney (BHK) cells, and probably others, support efficient replication when transfected with authentic astrovirus genomic RNA or with *in vitro* transcribed full-length astrovirus RNA, although they are not easily infected, suggesting that barriers at the entry level could determine the susceptibility of some cells to virus infection (22). The hepatic Huh7.5.1 cell line is an excellent candidate for rescue virus after viral RNA transfection, since they are at least as permissive as CaCo-2 cells for virus infection, but more efficiently transfected (21).

To efficiently infect cells, HAstVs must be activated by treatment of the virus with trypsin. Cellular receptors have not been identified, but the viral spike structure suggests that the receptor-binding site has an amino acid composition characteristic for polysaccharide recognition (14). HAstVs enter cells by endocytosis through a clathrin-dependent mechanism (36, 37). Upon cell infection, the gRNA is translated into the nonstructural proteins. These viral proteins use the gRNA as a template to synthesize a full-length negative-sense, antigenomic RNA (agRNA), which, in turn, is used as a template to produce the two positive-sense viral RNAs, the gRNA and a sgRNA, which are colinear at their 3'-ends (38). Conserved sequences at the 5'-end of the genome, just upstream of the transcription initiation site of the sgRNA, suggest that the sgRNA is synthesized from an internal promoter in the negative sense RNA (39, 40). All three forms of RNA are produced with similar kinetics although their relative proportion is different; the sgRNA is about 5-fold more abundant than the gRNA, while the agRNA abundance ranges from 0.7% to 4% of that of gRNA (38, 41). The production of sgRNA is affected by sequence

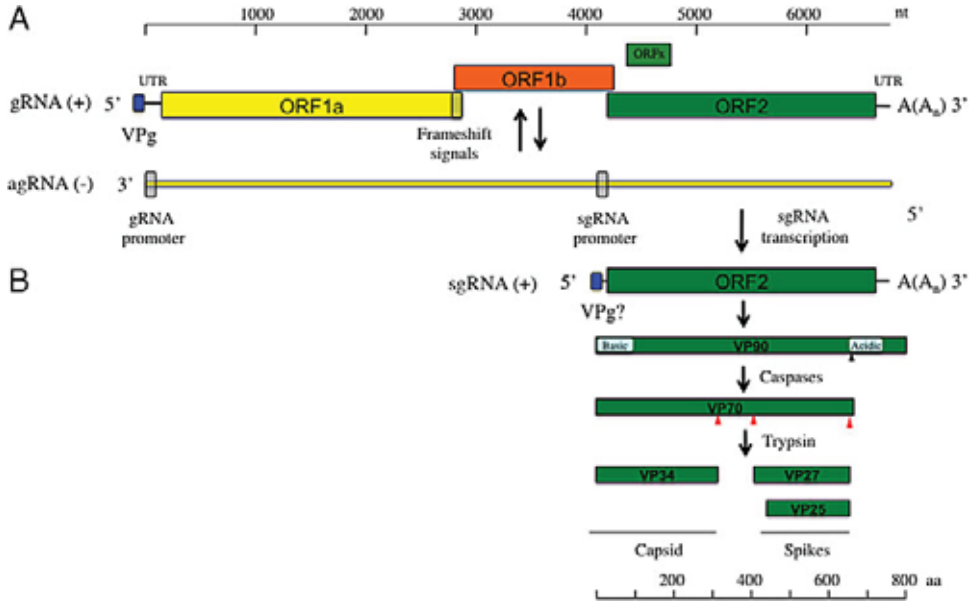

FIGURE 3 Astrovirus genome replication and transcription and synthesis of the structural proteins. (A) The genome of astrovirus (shown here for HAstV-8) is used as template to synthesize the negative-sense antigenomic RNA (agRNA), which, in turn, serves as template to synthesize the full-length positive sense genomic (gRNA) and subgenomic (sgRNA) RNAs. The sgRNA is about 2.4 kilobases and has a polyA tail at the 3'-end and likely also has a VPg protein covalently bound at its 5'-end. (B) The sgRNA is translated into the structural polyprotein precursor VP90, which contains an N-terminal conserved domain and a C-terminal hypervariable domain. The conserved domain has a basic amino acid region that is thought to interact with the viral RNA and a second region that has been predicted to be the shell of the viral capsid. The hypervariable domain conforms the viral spikes, and contains an acidic region towards the carboxy terminus of VP90. The processing pathway of this precursor protein has been described in detail (3). Briefly, VP90 is assembled into particles, and the acidic region at its carboxy terminus is processed to yield a protein of 70 kilodaltons (VP70) (43). This processing is carried out by caspases, cellular proteases activated during virus infection that are involved in cell death by apoptosis. The caspase cleavages correlate with, and are required for, the release of virions from the cells (43, 44). The released virions formed by VP70 are poorly infectious, and full activation of their infectivity requires that VP70 is cleaved by extracellular trypsin. Processing of the VP70-containing particles by this enzyme is sequential, producing final protein products of 34, 27, and 25 kilodaltons through at least eight intermediary polypeptides (3). The black arrow head represents a site for caspase cleavage and the red arrow heads, sites for trypsin cleavage. The intermediate caspases and trypsin cleavage products are not shown but can be found in other publications (28, 40).

changes in the hypervariable domain of the carboxy-terminal region of the nsp1a protein (23).

Astrovirus replication, as with other positive-sense RNA viruses, seems to occur in association with membranes, since the viral structural and nonstructural proteins, including the RNA-dependent RNA polymerase, as well as the gRNA and agRNA and infectious virus, are associated with membranes and colocalize with calnexin [(25, 42), Murillo A et al, submitted for publication]. Recently, the cellular proteins present in membranes, to which astroviral proteins and RNA associate, were determined by LC-MS/MS. Functional analysis of the protein-protein interaction network showed some biological processes that were enriched in these membranes, such as gluconeogenesis, fatty acid beta-oxidation, fatty acid synthesis, long chain fatty acid synthesis and tricarboxylic acid cycle (Murillo A et al, submitted for publication). In astrovirus-infected Caco-2 cells, VP90 assembles intracellularly into viral particles and is then processed by cellular caspases to yield VP70 during virus cell egress through a mechanism that does not involve cell lysis. Extracellular virions grown in cells in the absence of trypsin are composed by VP70 (43, 44).

Stability/Inactivation

Astrovirus particles are stable at a wide pH range (3 to 10) (45) and tolerate exposure to detergents, such as octyglucoside (20) and lipid solvents. HAstV infectivity is affected minimally if the virus is incubated at 4°C for 45 days in drinking water, although the titer drops by about two log10s when the virus is maintained at 20°C (46). Temperatures of 60°C for more than 5 minutes drastically reduce infectivity. At -70 to -85°C the virus infectivity is retained for several years, although with repeated freezing and thawing infectivity decays. The stability of the infectivity of HAstV is lower in surface water than in groundwater, and the positive correlation between the detection of the gRNA and infectivity suggests that the detection of genetic material could be used to determine water contamination with infectious HAstV (47). Another indicator of the infectivity of HAstV particles is the amount of oxidative damage they have experienced (48).

EPIDEMIOLOGY

Distribution, Incidence, and Prevalence of Infection

HAstVs have been isolated from fecal samples of patients with gastrointestinal disease from all around the world. HAstV infections are recognized mainly in young children, elderly people and immunocompromised patients, although infections in healthy adults can occasionally occur. HAstVs

are an important cause of viral gastroenteritis in young children (mostly under five years of age), second only to rotavirus, although in the last years a clear increase in the detection of noroviruses and adenoviruses has been noted (9). About 75% of children older than 10 years have antibodies to astrovirus. With the use of sensitive molecular methods for diagnosis, the incidence of astrovirus infections has been estimated at 2 to 9% in children with gastroenteritis (9, 49, 50), as compared to less than 2% in healthy children. These rates were previously underestimated mainly due to the less sensitive methods employed. Isolated studies have reported prevalences of HAstV as high as 30% to 61% in children with gastroenteritis (51, 52). Novel HAstV strains have been found in multiple stool samples collected around the world (53), but their correlation with gastroenteritis is not clear and, in one report, HAstV MLB1 was more prevalent in healthy individuals than in patients (54). MLB1 seropositivity was high in children and was estimated as 100% in adulthood, suggesting that MLB1 infection is common (55). Seropositivity for VA-1/HMO-C, depending on the age, was between 20 and 36% in children and 63% in adults (56).

HAstVs have been associated with outbreaks in daycare centers for children (57, 58) and adults (59), in schools and residential institutions (60), and with neonatal gastroenteritis in hospitals (61). Some of the largest outbreaks were observed in Japan, where more than 4,700 primary and junior high school students and staff were affected in a single outbreak (62). HAstV VA-1 was associated with an outbreak of acute gastroenteritis in a childcare center (63). Outbreaks in aged care centers showed attack rates between 12 and 100%, with serotype 1 strains being the most common (59).

In immunodeficient patients, the frequency of astroviruses reported as a cause of gastroenteritis is variable. Some studies report HAstVs as the principal viral cause of diarrhea in HIV-positive patients (12% in patients with diarrhea versus 2% in patients without diarrhea) (64, 65). Yet other studies suggest other viruses as the main cause (66). HAstVs can establish persistent infections in the immunocompromised host (67, 68).

Serotype Prevalence

As noted above, eight HAstV serotypes have been reported, based on neutralization assays with polyclonal antisera. Studies from different countries have shown that HAstV strains of serotype 1 are the most frequently found (in about 50% of the astrovirus-positive samples) (50), although other serotypes, like serotype 2, have been the most common in some studies (69, 70). Strains of serotype 7 have been rarely found (50). The second most prevalent serotype has varied depending on the particular study (71). Only a few epidemiological studies of novel HAstVs have been performed to date and have found MLB1 or MLB3 as the more prevalent (72, 73). The genotype of novel HAstV is based on the full-length sequence of ORF2.

Seasonality

In most temperate climates HAstV infections are more frequent in winter, while in tropical regions astroviruses are more frequently detected during the rainy season (74). Also, high water flow in areas of poor water quality or sewage contamination is one of the causes of fecal contamination (75) and, therefore, of transmission of astrovirus and other enteropathogens. In a study describing the seasonality of novel HAstVs, the highest frequency was observed from March to July (73).

Transmission

Contaminated food or water is the most frequent source of astrovirus infections. Large outbreaks of gastroenteritis in different countries (76) and sequence analysis of the astrovirus strains present in water supplies and fecal samples from hospitalized patients in South Africa have confirmed that food and water are important sources for virus infection (77). Classical HAstVs have been detected in sewage samples around the world (78–86), and recently HAstV MLB was found by deep sequencing in sewage samples from United States, Spain, Ethiopia (87), and Uruguay (88). HAstV is able to survive on inert materials for long periods, and it has been suggested that it can also be transmitted through fomites in nosocomial infections (46).

In general, astroviruses have a limited host range; however, the identification of novel astroviruses in human populations, which are genetically more closely related to animal than to classical human astroviruses, suggests interspecies transmission; zoonotic events have also been suggested (40, 51, 89). In support of this idea, recombination between human and both porcine (90) or marine mammal strains has been reported (91). People who have contact with turkeys can develop serological responses to turkey astrovirus, but whether this is the result of viral infection was not discerned (92).

PATHOGENESIS AND IMMUNE RESPONSE

Pathogenesis

HAstV has a specific tropism for epithelial cells of the small intestine. However, there is limited information on the pathogenesis of HAstV infection. Histopathological studies of biopsies from an immunodeficient patient showed that astrovirus infections are limited mainly to epithelial cells in the small intestine, particularly the jejunum, although the duodenum was also affected (Fig. 4) (68). Studies with this patient, as well as with turkeys infected with a homologous astrovirus (93), indicate that inflammation is not central to illness pathogenesis. The fact that astrovirus induces apoptosis in cultured cells of human origin (43, 94) suggests that this programmed cell death, frequently associated with a very minimal inflammatory response, could play a role in disease pathogenesis. With turkey astrovirus infections, virus can be recovered from different organs, although the small intestine seems to be the only organ where the virus replicates (95). Histological studies with tissues from mammals infected with astrovirus, which are probably more similar to human infections, indicate that the virus is localized in the epithelial cells, as well as in subepithelial macrophages of the small intestine. Intestinal epithelial cells are the primary site of astrovirus replication in humans, reflecting the high quantities of virus particles present in fecal samples. Up to 10^{10} astrovirus particles may be shed per milliliter of stool (23), although many samples contain much lower amounts of virus. Virus can persist in fecal samples of infected children up to two weeks after symptoms disappear, but this period frequently is much longer in immunodeficient patients with chronic infections (74). Gnotobiotic lambs, infected with ovine viruses indicate that the incubation period of the disease is less than 2 days, and they develop diarrhea that ends four days postinoculation (96). In this animal model, infection occurred primarily in the dome of epithelial cells overlying jejunal and ileal Peyer's patches, and virus secretion occurred between days 2 and 9 postinoculation. In humans, the median incubation period in adults was 4.5 days,

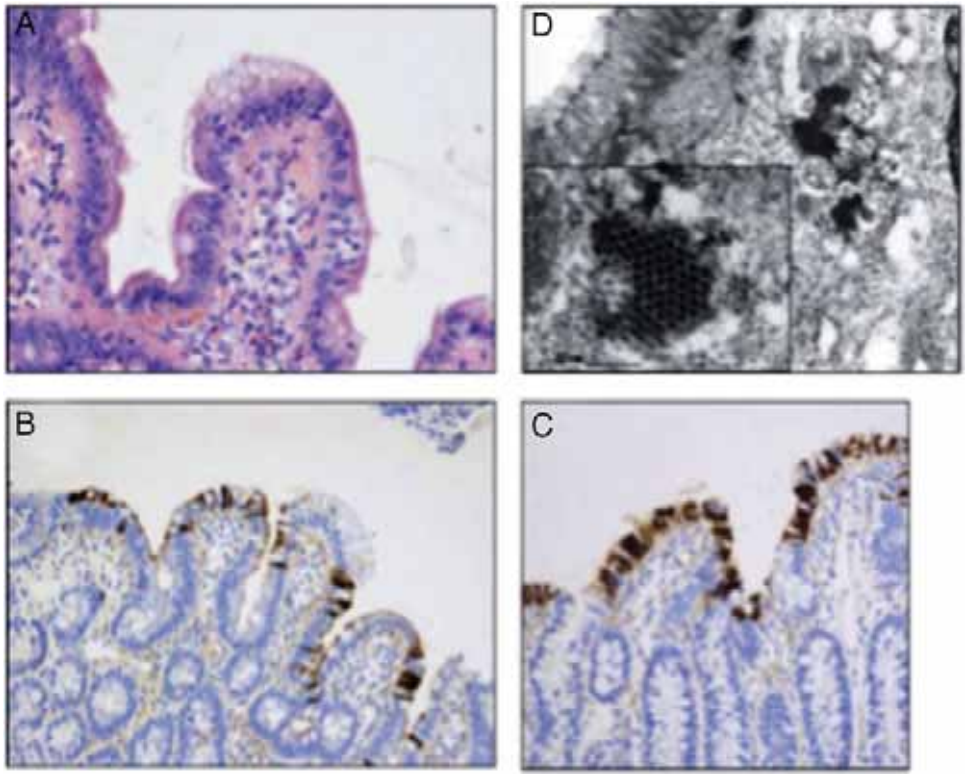

FIGURE 4 (A) Photomicrograph of a jejunal biopsy specimen from a bone marrow transplant recipient with astrovirus infection demonstrating villus blunting, nonspecific alterations in surface epithelial cells and a mixed lamina propria inflammatory infiltrate but without the presence of viral inclusion bodies (original magnification, x100). Also shown are photomicrographs of duodenal (B) and jejunal (C) biopsy samples from a bone marrow transplant recipient with astrovirus infection immunostained with anti-astrovirus antibody and demonstrating progressively extensive staining of surface epithelial cells, most commonly near the villus tips (original magnifications, x40 and x100, respectively). (D) Electron micrographs of a jejunal enterocyte demonstrating cytoplasmic paracrystalline viral arrays of astrovirus (original magnifications, x32,000 and x100,000 [inset]). (Reprinted from reference 68 with permission.)

with 75% of cases becoming symptomatic by 5.3 days (97). The capsid protein could be involved in the pathogenesis mechanism of astrovirus, since it induces a reduction in the transepithelial resistance in cultured cells (98).

Astroviruses have been shown to be potential neurotrophic pathogens in mink (SMS-AstV) and cattle (BoAstV CH13 and BoAstV NeuroS1). Viruses were detected in brain of cattle with a clinical neurologic disorder and in mink having a neurological disease, termed shaking mink syndrome (SMS) (99–101). In humans, the nonclassical HAstV VA-1/HMO-C has been found in neuronal tissue of patients with encephalopathy (Fig. 5), and this virus groups phylogenetically with SMS-AstV, BAstV CH3, and BoAstV NeuroS1. On postmortem examination, the patient's brain displayed features consistent with viral infection, since viral antigen and viral RNA were detected in glial (102) or neuronal cells (103, 104), while the viral RNA was absent or reduced in stool and intestinal tissue. Only one fatal case of classical HAstV has been reported in a patient with severe combined immunodeficiency. In this case viral RNA was found in stool but only in low levels in the brain. The patient had liver and lung failure, convulsions and evidence of meningoencephalitis (105). In all the cases where HAstV has been detected in brain tissue, the patients were immunocompromised.

The high prevalence of antibodies to HAstV VA-1/HMO-C suggests that the infection in humans is common but most frequently asymptomatic. Koch's postulates have not been established for HAstV VA-1/HMO-C and the neuropathology, since these viruses have not been adapted to grow in cultured cells, and currently animal models do not exist to reproduce the disease. However, data with the SMS-AstV can be reproduced by intracerebral inoculation of healthy animals with brain extracts obtained from ill individuals, and the viral RNA is only detected in diseased animals (naturally or inoculated) (99).

Members of the *Avastrovirus* genus are associated with different diseases in different species. Astrovirus isolated from ducks cause fatal hepatitis in ducklings (106), while isolates from chickens are associated with renal and gastrointestinal diseases (107). Turkey astroviruses cause not only enteritis but immunopathology that leads to a disease called poult enteritis mortality syndrome (108–110); in pigeons astrovirus has been related to nephritis (111).

Immune Response

The existence of several HAstV serotypes suggests that neutralizing antibodies exert an immune selection pressure on the virus. In adult volunteers HAstV seroconversion occurred after inoculation, and, in some cases, the antibodies recognized other serotypes, indicating that cross-neutralizing antibodies can be elicited upon infection (112, 113). The role of antibodies to control a natural infection is not clear, and it is not known if primary infections in childhood

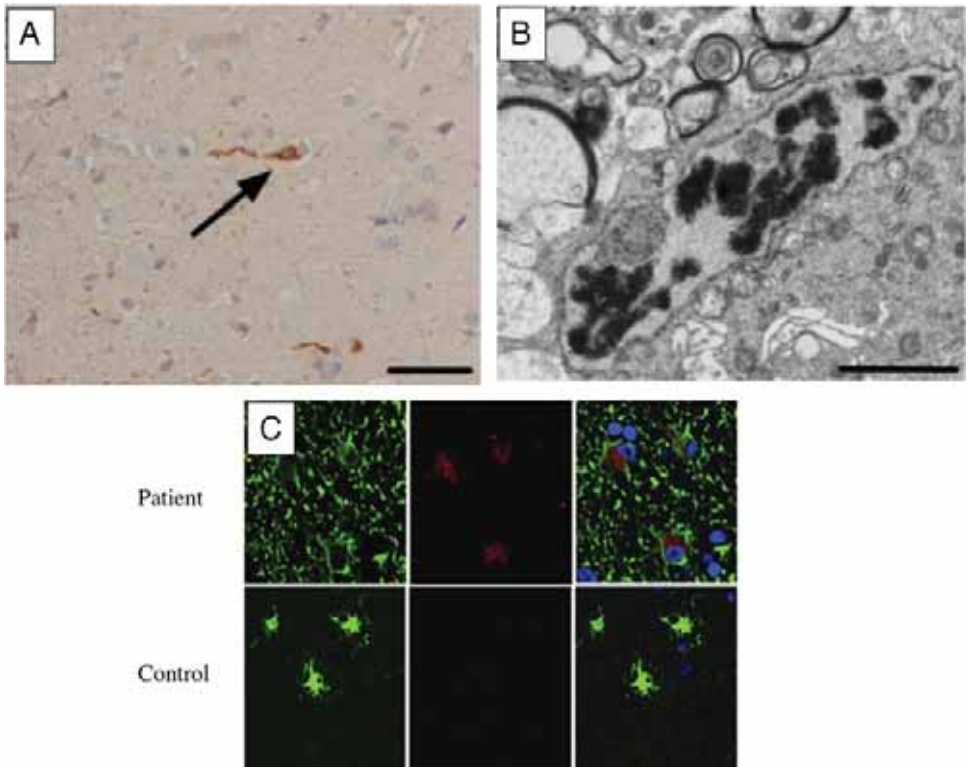

FIGURE 5 Detection of the capsid protein of HAstV VA-1 in neuronal tissue from immunosuppressed individuals. (A) Immunohistochemistry with an antibody against astrovirus shows extensive staining of cell bodies and processes in the neurophil. Some of the astrovirus-positive cells have the morphology of pyramidal neurons (indicated by an arrow; scale bar, 50 μm). (B) Electron microscopy of the same patient in (A) showed a rare focus of crystalline material but no viral particles (scale bar, 2 μm). (C) Indirect immunofluorescence of brain tissue sections from a 15-year-old boy with X-linked agammaglobulinemia and astrovirus encephalitis (patient), and a control patient with astrogliosis not caused by astrovirus infection (control). The sections were stained for the astrocyte marker glial fibrillary acidic protein (GFAP, green) and for viral capsid protein (red); the blue color (DAPI) indicates nuclear counterstaining. Original magnification, ×100. (Adapted from references 103 (A and B) and 102, with permission.)

provide heterotypic protection for subsequent infection, although symptomatic infections in elderly patients suggest that antibodies acquired early in life (114) do not provide protection from illness at a late age.

The mucosal immune system could be important in protecting individuals from repeated astrovirus infections. T cells that recognize astrovirus antigens in a human leukocyte antigen (HLA)-restricted manner were found to reside in the intestinal lamina propria of healthy adults (115). These HAstV–specific CD4+ T cells produced helper T-cell subtype 1 cytokines, interferon gamma and tumor necrosis factor when activated. Recent isolation of murine astrovirus and the use of mice defective in the production of T cells confirmed the role of adaptative immunity in the control of astroviruses, and the use of knockout mice for STAT-1 suggests that the innate immune response can also have a role in the immune control of these viruses (116). HAstVs induce the production of interferon, and viral replication is partially susceptible to it (117). The capsid protein of HAstVs inhibits the complement system (118).

CLINICAL MANIFESTATIONS

A typical astrovirus infection in humans is characterized by acute gastroenteritis, consisting of watery diarrhea for two to three days that may be accompanied by vomiting, fever, anorexia, abdominal pain, and a variety of constitutional symptoms that typically last no more than four days (74). Symptoms are similar to those caused by other gastrointestinal viruses like rotavirus and calicivirus, but are usually not severe enough to require hospitalization. Cases of severe astrovirus infections have been reported during infections with serotypes 6 (119) and 3 (120). HAstVs have been detected in children with intussusception, although astrovirus infection does not appear to be associated with increased risk of this problem (121–125). The severity of an astrovirus infection is associated with the immune status of the patient, since immunodeficient patients have shown persistent infections with an extended course of infection and viral shedding (64). Illness due to astrovirus infection may be more severe in the elderly, given the likelihood of general debility and underlying serious medical conditions in many of these patients (59). Death associated with astrovirus infection is extremely rare.

The clinical manifestations of infection with novel HAstVs are not clear, since some studies have associated them with gastroenteritis while others have detected the virus in the absence of gastroenteric symptoms. The patients in whom novel HAstVs have been detected in cerebral tissue had neuropsychiatric manifestations, including irritability, dystonia, reduced consciousness, withdrawal, ataxia, memory loss, headache, and vestibulocochlear dysfunction. Viremia was reported in one case, and the patient developed respiratory and gastrointestinal complications. The capsid

protein or viral RNA was detected in neurons and glial cells (Fig. 5). All encephalitis cases have proven fatal (102–104). One report found classical HAstV-4 in the brain. In this case no detection of viral antigen was reported, but viral RNA was detected in postmortem autopsy tissue from several organs including brain; the patient developed multiorgan dysfunction (105).

LABORATORY DIAGNOSIS

Virus Isolation

Caco-2 is the cell line of choice for virus isolation from human fecal samples (35, 126). Trypsin treatment of the sample is necessary to successfully adapt HAstV to cells. Since the cytopathic effect caused by an astrovirus infection is difficult to observe in most cases, especially in the first passages in Caco-2 cells, this technique should be complemented with additional methods to enhance specificity and sensitivity, including immunofluorescence with group-specific antibodies or RT-PCR EM of infected cells, which can also be used to confirm the isolation of astrovirus in culture. Notably, paracrystalline structures around the nucleus are formed by multiple particles (Fig. 1B). As mentioned earlier, T84 and PLC/PRF/5 hepatoma cells may also be used for isolation of human astroviruses directly from fecal specimens. Other cell lines, like HEK-293, HT29, MA104, and LLC-MK2, that have been used to grow cell culture-adapted astrovirus strains, are not easily infected with the virus present in fecal samples. The novel HAstV strains have not yet been grown in cell culture.

Electron Microscopy

EM has been used to distinguish this virus from other viruses of similar size; however, this technique requires qualified personnel to obtain reliable results, since a low percentage of particles in fecal samples show the star-like morphology. The EM detection method requires 10^6 to 10^7 particles per gram of stool (127). Sensitivity by EM can be improved by treating the sample with astrovirus-specific antibodies in order to agglutinate viral particles, facilitating detection.

Antigen Detection

Enzyme immunoassays (EIA) have been developed to detect the classical eight HAstVs in fecal samples. Variations of this assay have used monoclonal antibodies that recognize a group antigen, as well as serotype-specific polyclonal antibodies (128–131). EIA (e.g., ProSpecT Astrovirus Test, Oxoid Microbiological Products, UK) is useful to detect astroviruses in a large number of samples, showing specificities in the range of 90 to 98%. It has been estimated that a positive EIA requires at least 10^5 and 10^6 viral particles per gram of stool (127). The high antigenic divergence of avian viruses has not permitted the identification of antibodies to common epitopes to be used in immunoassays to detect all AstV that infect flocks. Similarly, no antibodies to common epitopes to detect the novel HAstV and other mammalian astroviruses have been produced.

Nucleic Acid Detection

Astrovirus nucleic acid can be detected in fecal samples by RT-PCR. The most useful oligonucleotide primers to detect serologically distinct strains of classical HAstVs were selected from highly conserved regions, such as the 5'-terminal region of ORF2, the 3'-end of the genome and the RNA-dependent RNA polymerase gene (132). However, oligonucleotide primers from the hypervariable region of ORF2 have also been used to genotype human isolates, since the amino acid sequence of this region correlates with the serotype (133). The sensitivity of RT-PCR (approximately 10 to 100 particles per gram of stool) (127) is higher than the sensitivity of EIA, although it can depend on several other factors, such as the primers used, the protocol to obtain the viral RNA and the reaction conditions. The sensitivity of RT-PCR to detect HAstV may be enhanced when it is combined with cell culture. This combined method has been successfully used to detect human astroviruses in water samples (134). Sequencing of the amplified DNA fragment by RT-PCR from a fecal sample can be useful to confirm astrovirus presence in that sample and to distinguish genotypes. RT-PCR assays have also been used to confirm the presence of astrovirus in animal samples.

For detection of both classical and novel HAstVs, primers that, in principle, could detect all strains have been designed, although no commercially available assays exist. After RT-PCR genome amplification with these pan-astrovirus primers, positive samples are amplified with specific primers, followed by DNA sequencing of the amplicon to determine the virus genotype (63). More recent developments include several protocols for real-time RT-PCR, several of which are designed as multiplex assays for detection of multiple pathogens (for instance, Astrovirus Assay, CeramTools, France; FilmArray, Biomérieux, France; Diagenode, Belgium; and Seeplex Diarrhea ACE Detection, Seegene, Korea) (9). The detection of astrovirus has also been reported using DNA microarray platforms (135–137). Deep sequencing was used in the identification of MLB and VA HAstVs and is a useful technique for identification of emerging astroviruses (10, 63).

PREVENTION

Prophylactic measures to avoid astrovirus spreading in the population are important but may be difficult to achieve. Sanitation is particularly important in institutions, such as daycare centers, aged-care centers, and hospitals with children and with immunocompromised patients, where outbreaks can emerge. Since astroviruses are resistant to a number of chemical treatments, appropriate disinfectants should be used. Soapy water and ethanol wipes were shown to reduce diarrhea due to astrovirus in a medical unit attending children with immune disorders (138). Standard water chlorination, although not totally effective, can help to diminish astrovirus viability (46, 47). Universal hygienic procedures must be used to prepare the food. No vaccines are available to prevent astrovirus disease in humans. In chickens, vaccination with the recombinant astrovirus capsid protein induced partial protection against the runting and stunting syndrome (139).

TREATMENT

As mentioned above, the gastroenteritis caused by astrovirus is not as severe as that caused by other viruses, and it is self-limiting. Generally, astrovirus infections do not require a specific therapy, other than rehydration. However, in immunodeficient patients with persistent infections, intravenous immunoglobulin administration has been used. Immunoglobulin therapy of immunocompromised patients with persistent astrovirus infection has resulted anecdotally in virus clearance and diarrhea elimination (140), but this treatment did not work for bone marrow transplant patients with

chronic diarrhea, even though this preparation was demonstrated to have antibodies to the homologous infecting astrovirus serotype (138). In this group, virus excretion was reduced in two patients when T-cell responses started to be detected after transplantation, suggesting that CD4+ T cells could be important to control virus replication either directly or through activation of B cells to produce antibodies (115, 138). No antiviral agents have been developed to control astrovirus infections, although synthetic flavonoids cause a dose-dependent reduction of viral antigen synthesis (141).

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Coronaviruses

J.S.M. PEIRIS

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The name coronavirus derives from the Latin word "corona," meaning crown or halo, and this refers to the "crown-like" fringe of projections seen on the surface of virus particles when viewed under the electron microscope (Fig. 1). The first coronavirus to be recovered was infectious bronchitis virus (IBV) from chickens with respiratory disease, reported by Beaudette and Hudson in 1937 (1). Murine hepatitis viruses (MHV) (2) and transmissible gastroenteritis virus (TGEV) in swine (3) were then recognized as causes of other animal diseases. The relationship between these viruses was not appreciated until after the human coronaviruses (HCoVs) were discovered in the 1960s and the *Coronavirus* genus was defined. Tyrrell and Bynoe (4) described the first HCoV, designated as B814, by inoculating specimens from a patient with a "cold" onto organ cultures of human embryonic trachea. Using electron microscopy (EM), the virus was found to resemble avian IBV (5). At about the same time, Hamre and Procknow (6) recovered five HCoV strains from medical students with colds and cultivated them in human embryo kidney cells. The prototype strain HCoV 229E had morphology that was identical to that of B814 and IBV. McIntosh et al. used the organ culture technique to recover six further strains, including the prototype strain HCoV OC43 and three other strains considered antigenically unrelated to either OC43 or 229E (7).

Until 2002, coronaviruses were perceived primarily as causes of the "common cold" and were not generally a subject of major public health or research interest. In the winter of 2002–2003, an unusual and often lethal form of pneumonia, a disease subsequently named severe acute respiratory syndrome (SARS), appeared in the Guangdong Province of China. Within days of this disease spreading to Hong Kong in late February, international air travelers seeded outbreaks in Vietnam, Singapore, Toronto in Canada, and elsewhere. By the end of this brief but global epidemic in July 2003, 8,096 cases had been recorded, 744 of them fatal, in 29 countries across five continents. Transmission within healthcare settings was a notable feature, accounting for 21% of all cases (reviewed in reference 8).

The resurgence of scientific interest in coronaviruses following the emergence of SARS, supplemented in part by the development of new culture-independent molecular methods for detection and identification of viruses, led to the discovery of three other coronaviruses causing disease in humans: NL63, HKU-1, and in 2012, Middle East respiratory syndrome coronavirus (MERS-CoV) (9–11). HCoVs 229E, OC43, NL-63, and HKU-1 (collectively referred to as endemic HCoVs below) are endemic in the human population and are distributed globally. SARS-CoV is no longer circulating within the human population, but MERS-CoV continues to cause sporadic zoonotic cases, sometimes followed by clusters of transmission between humans, mainly within healthcare facilities or families.

VIROLOGY

Classification

Coronaviruses have been classified as members of the order Nidovirales, positive-sense, single-stranded RNA viruses that replicate using a nested ("nido") set of mRNAs. The family Coronaviridae contains two genera, *Torovirus* and *Coronavirus*. The original basis for classification of the coronaviruses into a separate genus lay in the distinct morphology of the members (12) (Fig. 1). This classification subsequently has been justified by genetic relatedness and similar strategies of replication. The *Coronavirus* genus is a large one, with representative viruses infecting multiple species, including chickens, turkeys, ducks, geese, other birds, rodents, cats, dogs, rabbits, cattle, horses, antelopes, camelids, pigs, aquatic mammals (dolphins, whales), bats, and humans. Many of the animal coronaviruses are of great economic importance. On the basis of genetic homologies, the coronaviruses are divided into alpha, beta, gamma, and delta coronavirus groups (Fig. 2). As defined by the International Committee on Taxonomy of Viruses, a virus is grouped into a known genus if the genetic sequence of RNA-dependent RNA polymerase, nonstructural protein (nsp) 5, nsp12, nsp13, nsp14, nsp15, and nsp16 share more than 46% nucleotide sequence homology. Previously, alpha-, beta-, and gammacoronaviruses had been designated as group 1, group 2, and group 3 coronaviruses, respectively, with deltacoronaviruses being a more recently defined group. Alphacoronaviruses include HCoV 229E and NL63. Betacoronaviruses are subdivided into groups A–D, with HKU1 and OC43 included in group A, SARS-CoV in group B and MERS-CoV in group C. Gammacoronaviruses are largely avian in origin and include IBV as well as marine mammalian viruses, whereas deltacoronaviruses include

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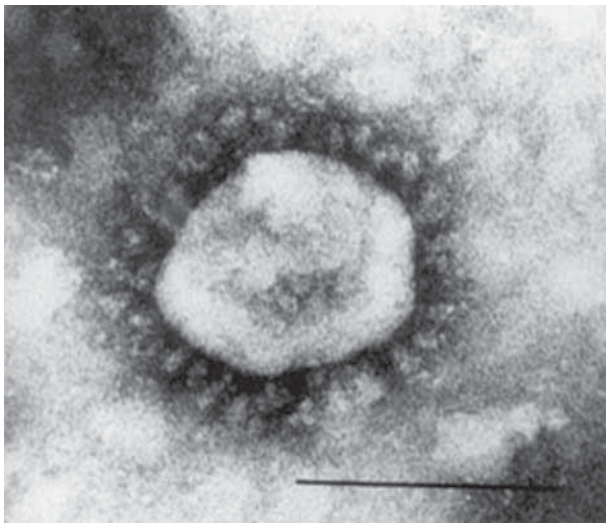

FIGURE 1 Coronavirus OC16, viewed by EM and negatively stained. The characteristic round or oval shape is seen, along with petal-shaped peplomers. Bar, 100 nm.

viruses of avian species and swine. There is a large diversity of bat coronaviruses within alpha- and betacoronaviruses, and thus bat coronaviruses are believed to be ancestral to all alpha- and betacoronaviruses. Rodent coronaviruses appear to be intermediate ancestors of the betacoronavirus group A. Similarly, avian coronaviruses are believed to be ancestral to gamma- and deltacoronaviruses (13).

Several coronavirus species cause gastroenteritis in newborn or young animals, and it was therefore not surprising when coronavirus-like particles (CVLPs) were found by EM in human feces. The identity of CVLPs in human intestinal contents and their role in disease are still unresolved. Some of the confusion about the role of enteric coronaviruses as causes of diarrhea may be related to the similar appearance of toroviruses in negatively stained stool specimens examined by EM. Toroviruses, belonging to a separate genus within the family Coronaviridae, are well-characterized causes of diarrhea in calves and horses. Human toroviruses, partially purified from stool samples, have been shown to be serologically related to both equine and bovine toroviruses (14, 15) and to contain sequences at the 3' end almost identical to those of equine torovirus (15, 16). With the help of serologic specificity, EM identification, and genetic characterization, differentiation from coronaviruses is possible (16). Likely the distinct roles of toroviruses and enteric coronaviruses will be clarified in the future.

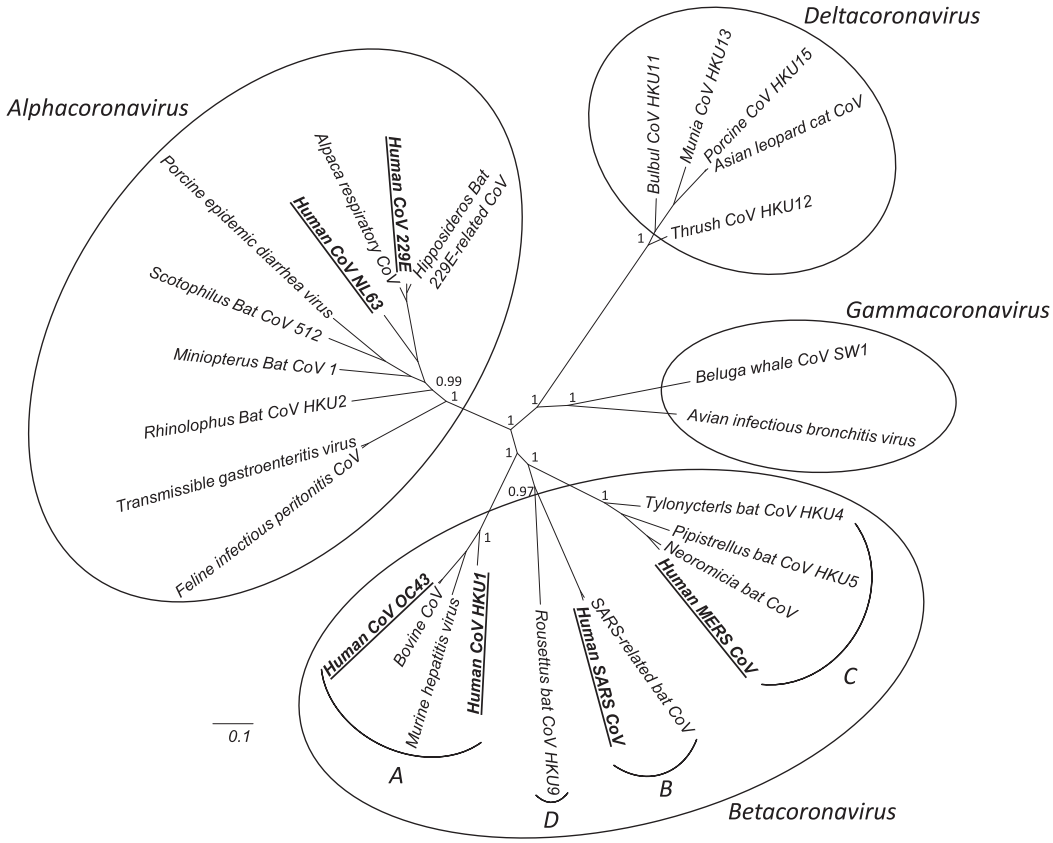

FIGURE 2 Phylogenetic relationships among coronaviruses. The phylogenetic tree was generated with an RNA-dependent RNA polymerase gene using the maximum likelihood method. Supports for branches were estimated by the Shimodaira-Hasegawa-like method and are shown at major nodes. The four coronavirus genera are indicated in circles. The betacoronavirus lineages A–D are denoted. Human coronaviruses included in the analysis are underlined.

Composition of Virus

Virion Structure

Coronavirus virions are round, enveloped, moderately pleomorphic, medium-sized particles measuring 100–150 nm in diameter and covered with a distinctive fringe of widely spaced, club-shaped surface projections (Fig. 1) (17). The projections are about 20 nm in length and represent the spike (S) protein, which aggregates in trimers to form the characteristic peplomers of the virus. Some members of betacoronaviruses, including OC43, also contain a shorter envelope protein, named hemagglutinin esterase (HE).

In EM thin sections of infected cells, virus particles have a diameter of 85 nm, a typical bilayer external membrane, and a coiled nucleic acid core that is, in cross section, 9–11 nm in diameter. These particles have been observed to bud from the membranes of the Golgi apparatus or endoplasmic reticulum (ER) of an infected cell and to accumulate in cytoplasmic vesicles (18) (Fig. 3). Infected cells often have particles on the cell surface that represent virus disgorged from cytoplasmic vesicles, rather than budding of virus at the plasma membrane.

Unlike coronaviruses, which are round in shape, toroviruses have a doughnut shape. They have few 20-nm spikes but have a fringe of smaller spikes, 7–10 nm in length (19, 20).

Genome

The genome of coronaviruses is the largest known RNA virus genome, 25.4–31.8 kb in size. It is single stranded, positive sense, capped, and adenylated. The order of genes is shown in Fig. 4A and is roughly identical throughout all coronavirus species, namely, 5′-replicase–spike (S)–envelope (E)–membrane protein (M)–nucleocapsid (N)-3′. In those species containing the HE gene, this is found between the replicase gene and the S protein gene. Many species have additional genes that code for accessory proteins, the presence of which can vary in different viruses (Fig. 4A).

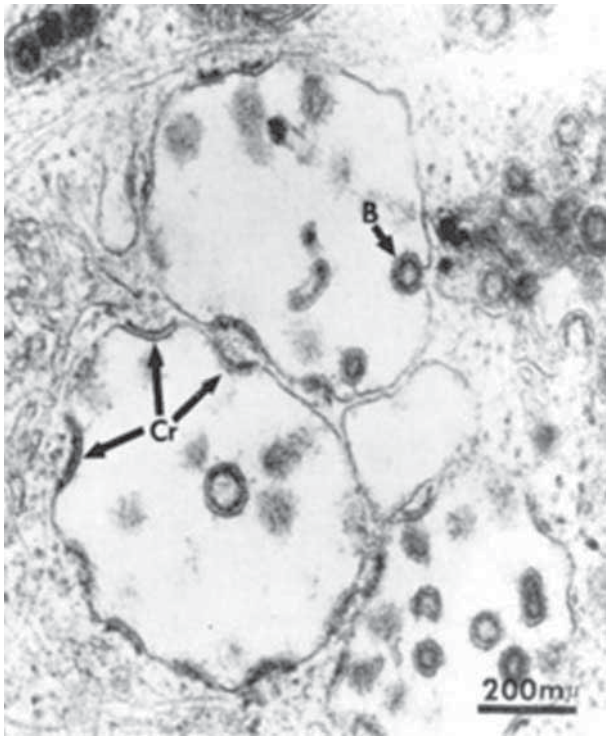

FIGURE 3 Coronavirus 229E in WI38 cells. Characteristic crescents (Cr) of budding particles (B) are seen, as well as particles that are free in cytoplasmic vesicles.

Major Structural and Regulatory Proteins

The large surface glycoprotein, the S protein, is oriented with its amino terminus facing outward, is N-glycosylated, assembles into trimers, and forms the club-shaped surface projections. The S protein is a prototypical class 1 fusion protein and is involved in receptor binding and fusion functions. Following cleavage by cellular proteases into S1 and S2 subunits, the former is involved in interaction with receptors whereas the latter is involved in fusion of the viral and cellular membranes. Coronavirus cell and tissue tropism, disease pathogenesis, and host range are initially controlled by interactions between the S1 subunit and host cell receptors; however, proteolytic activation of the spike protein by host cell proteases also plays a critical role. A number of host proteases have been shown to process the spike protein proteolytically, including, but not limited to, endosomal cathepsins, cell-surface transmembrane protease/serine (TMPRSS) proteases, furin, and trypsin (21). The spike protein elicits neutralizing antibody responses.

The HE glycoprotein, found on the surface of some betacoronaviruses, is genetically related to a similar protein in influenza C virus. The esterase function may have a role in the release of virus from infected cells. Embedded in the membrane of the virus particle is the M protein, a 20- to 35-kDa glycosylated protein that penetrates the viral envelope three times and has a key role in virus assembly, probably interacting with the RNA–nucleoprotein complex of the virus during the maturation of the particle. Also present in the membrane is a sparsely represented protein, the E protein. The nucleoprotein itself is a 50- to 60-kDa phosphoprotein, which binds to and presumably stabilizes the positive-strand RNA of the virus.

The open reading frame 1a/b (ORF1a/b) of the coronavirus genome encodes a huge polyprotein that is cleaved by cellular and viral proteases into some 16 nonstructural proteins, including an RNA-dependent RNA polymerase, several RNases, several proteases, and several other essential proteins. Unlike other RNA viruses, which lack proofreading mechanisms to correct mutations that occur during replication of RNA, coronavirus nonstructural protein 14 provides proofreading activity (22). Coronaviruses have a number of accessory proteins, some of them not essential for virus replication *in vitro* but important *in vivo*. Some of these, for example, ORF6, ORF3b, and ORF4a, are interferon antagonists that help the virus evade host innate immune responses (reviewed in 23, 24).

The proteins of enteric HCoVs have not been well characterized, although it appears that their size and number are similar to those of other coronaviruses (25).

Biology

Replication Strategy

The biology of coronaviruses (reviewed in 25, 26) is complex. Coronaviruses bind to cells through receptors, although the details are not presently known for all members of the genus. HCoV 229E binds specifically the metalloprotease human aminopeptidase N (27), whereas the alphacoronavirus HCoV NL63 and betacoronavirus SARS-CoV bind specifically to another metalloprotease, angiotensin-converting

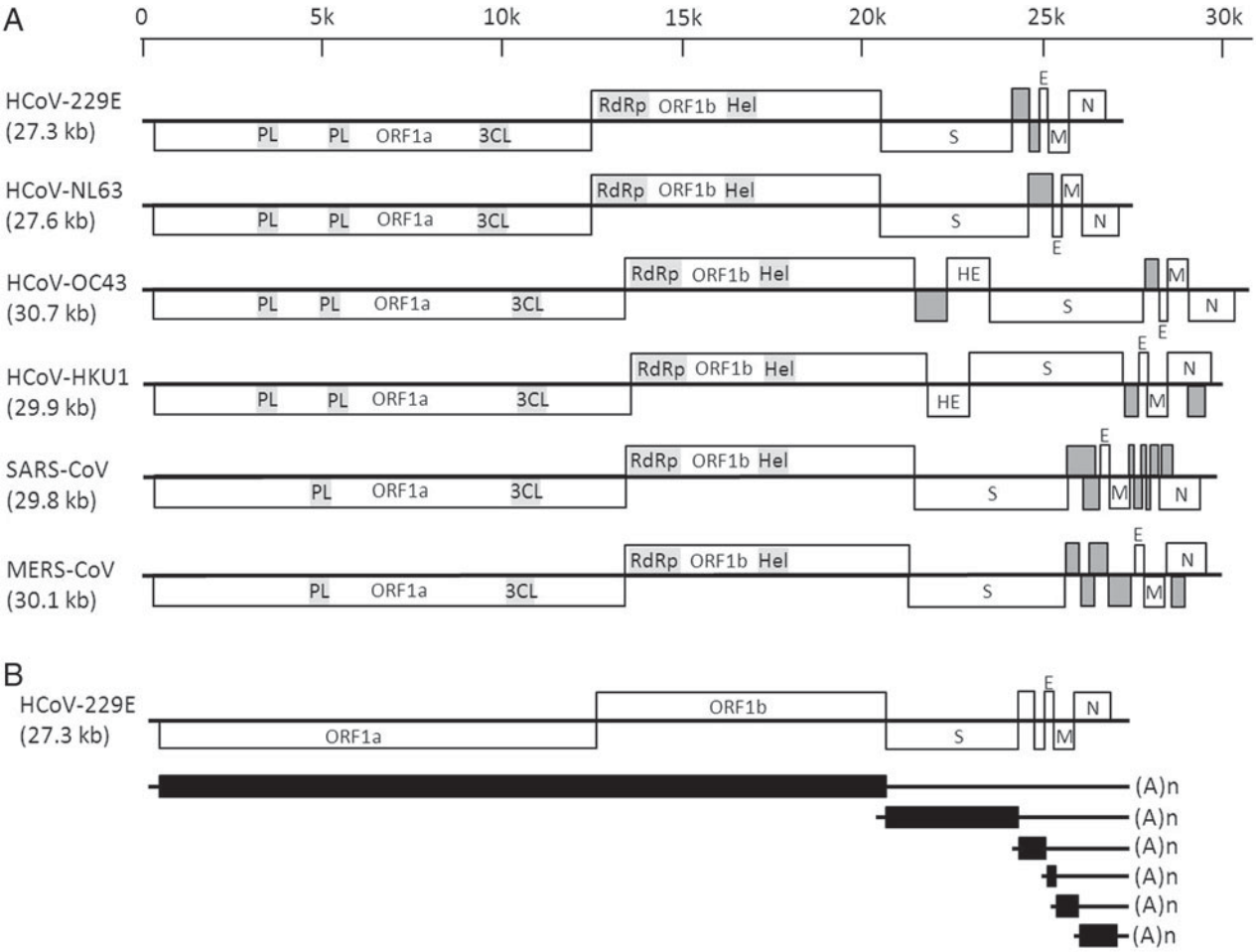

FIGURE 4 (A) Genome organization of alphacoronaviruses HCoV 229E and HCoV NL63 and betacoronaviruses HCoV OC43, HCoV HKU1, SARS-CoV, and MERS-CoV. Genes and open reading frames (ORFs) of the genomes are shown, with accessory genes indicated in dark grey boxes. Positions of papain-like proteases (PL), chymotrypsin-like protease (3CL), RNA-dependent RNA polymerase (RdRp), and helicase (Hel) encoded in ORF1a and ORF1b are shown in light grey. (B) The 3' coterminal nested set of coronavirus mRNA is shown for HcoV 229E. The black boxes indicate the region of each mRNA being translated.

enzyme 2 (ACE2) (28, 29). Betacoronavirus MERS-CoV binds to dipeptidyl peptidase-4 (DPP4; CD26), which is found on many human tissues including the respiratory epithelium (30), whereas MHV uses carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM-1) as a receptor (31). Betacoronaviruses can also use both S protein and (if present) HE to bind to 9-*O*-acetylated neuraminic acid molecules on many biological membranes as an additional receptor. HKU1 binds *O*-acetylated sialic acid residues on glycoproteins to initiate the infection of host cells (32), but this sialic acid is not the 9-*O*-acetylated neuraminic acid molecule that binds OC43 and bovine coronaviruses.

Viral entry is accomplished through fusion of the plasma and viral membranes at the cell surface or by receptor-mediated endocytosis via a clathrin-dependent mechanism. Once in the cytoplasm, the genomic viral RNA is translated by host machinery to produce the polyprotein that is then cleaved by virally encoded papain-like protease and the main protease to produce (among other proteins) an RNA-dependent RNA polymerase. This enzyme helps make a minus-sense copy of the full-length genome and also a set of nested minus-strand RNAs from the genomic RNA, which serve as templates for mRNA synthesis (Fig. 4B). Each of the nested-set mRNAs begins with a leader sequence, identical to the leader sequence found at the 5′ end of the full-length genomic RNA, then an intergenic sequence, then the translated ORF, and then all bases through to the 3′-polyadenylated end. Thus, all of the mRNAs except the smallest, that coding for the N protein, are polycistronic, containing sequences coding for more than one protein, although only the first cistron in line is actually translated during protein synthesis (25, 26).

The various viral proteins are synthesized, processed, and transported by cellular cytoplasmic machinery. Coronaviruses can replicate in enucleated cells. The S protein and HE are cotranslationally N-glycosylated in the ER and processed in the Golgi apparatus where the S protein is oligomerized into a trimer. The S protein undergoes proteolytic cleavage either intra- or extracellularly, as mentioned above. The M protein is inserted into the ER shortly after synthesis and accumulates in the Golgi apparatus.

Assembly takes place when the N protein binds to genomic RNA and recognizes signals on the M protein in the ER or the Golgi apparatus. The S protein and HE are in-

corporated into the ER and Golgi membranes at the time of budding, and viruses accumulate in large numbers in smooth-walled cytoplasmic vesicles. These fuse with the plasma membrane and virus is released.

Host Range and Zoonotic Emergence

The host range of coronaviruses is largely determined by the viral spike–host cell receptor interactions and the dependence of viral spike activation by defined host proteases (see above). Coronaviruses rival influenza viruses as a group where human epidemics or pandemics can arise from animal viruses. Four (HCoV 229E, HCoV OC43, MERS-CoV, SARS-CoV) of the six currently known HCoVs originated by zoonotic transmission events within the past few hundred years (33) (Fig. 2). Ancestral viruses to HCoV 229E exist in African bats and camelid species, suggesting that 229E arose from bat viruses with camelids as a potential intermediate host (Fig. 2) (34, 35). This pathway of emergence is reminiscent of zoonotic MERS-CoV infections, which currently appear to be transmitted from dromedary camels (36, 37), but it is possible that the ancestral viruses again reside in bat species (33, 38). HCoV OC43 arose from bovine coronaviruses (BCoVs), which have also crossed to other animal species, including other ruminants and camelids (33). SARS-CoV emerged from *Rhinolophus* bats (39). Intermediate hosts, such as civet cats within game animal markets in Guangdong, served as amplifiers for the virus, and these venues provided the interface for repeated human exposure (40). Other examples of interspecies transmission between animals include the emergence of canine CoV (CCoV) II and feline CoV II from recombination events between canine, feline, and porcine coronaviruses and CCoV II, which led to the emergence of TGEV (reviewed in 23, 41).

There are examples of dramatic changes of tissue tropism, pathogenesis, and virulence associated with genetic changes in the spike protein. TGEV, a cause of a virulent gastrointestinal disease of swine, had tropism for the intestine. Viruses with deletions of the spike protein gene of TGEV emerged spontaneously, leading to a change in the tropism of the virus to the swine respiratory tract. This virus, now called porcine respiratory coronavirus, causes a milder disease (41, 42).

Experimental Animal Models

Animal models are important for the investigation of viral pathogenesis, transmission, and the efficacy of treatments and vaccines. Successful experimental animal models should mimic the human disease; share comparable disease severity, increased in relevant demographic groups (e.g., age); manifest comparable pathology, virus tropism, and receptor distribution; and mimic the natural route of transmission. Experimental models for the endemic HCoV have not been investigated intensively, whereas experimental animal models for SARS and MERS have been studied (reviewed in 43, 44, 45). Nonhuman primates (NHPs), mice, hamsters, and ferrets have been investigated for this purpose.

Experimental infection of cynomolgus macaques and African green monkeys with SARS-CoV led to variable disease severity. In parallel with the demographics of disease in humans, aged macaques manifest more severe disease (see section on pathogenesis). Common marmosets (*Callithrix jacchus*) develop multiorgan disease. Experimental infection of mice leads to virus replication without overt disease, but disease severity can be increased by using older mice (46), using transgenic mice expressing the human ACE-2 receptor, or adapting the virus to mice by serial virus passage. The virus replicates to high titers in hamsters without overt disease. SARS-CoV infection of ferrets has been reported with conflicting results.

MERS-CoV infection of macaques is associated with viral replication and lung infiltration but mild disease. Infection of marmosets is associated with higher levels of virus replication and more severe disease; however, the intratracheal route of infection required has led to controversy regarding the biological relevance of this experimental model. Mice are not susceptible to MERS-CoV because mouse DPP4 does not support viral attachment and replication. Transduction of human DPP4 into mouse lung via an adenovirus vector makes mice susceptible to experimental challenge, leading to an interstitial pneumonia, but not to death. Aged mice have more severe disease, but still without virus-associated mortality. Transgenic mice with global expression of human DPP4 are permissive to robust viral replication, leading to severe respiratory and generalized disease and death. Hamsters and ferrets are not susceptible to MERS-CoV infection. Dromedary camels are a natural host for MERS-CoV, and infection causes minimal overt disease. In parallel, experimental intranasal infection of dromedaries is associated with viral replication and shedding in the nasopharynx associated with a mild nasal discharge. There is no evidence of virus dissemination (reviewed in 44).

Growth in Cell Culture

None of the HCoVs grows easily in cell culture without adaptation by passage. Strains related to 229E can be grown in primary or secondary human embryonic kidney cell lines, in diploid human fibroblast lines, and in a few heteroploid lines (47, 48). The most sensitive cell line for isolation of 229E from clinical specimens appears to be the diploid intestinal cell line MA-177 (48). The hepatoma line HUH7 has been recently used with success for isolation of HKU1, OC43, and 229E from clinical samples (49–51). LLC-MK2 and Vero B4 cells have been helpful in isolation of NL63, although the cytopathic effects (CPE) are somewhat nonspecific (52). The highest titers of both 229E and OC43 have been obtained by growth in human rhabdomyosarcoma cells (53). Plaque assays for HCoV-229E can be performed in human diploid fibroblasts (54), and those for both 229E and OC43 can be performed in rhabdomyosarcoma and fetal tonsil diploid cells (53).

Culture of HKU1 has also been possible in primary human tracheal–bronchial epithelial cells cultured in an air-liquid interface (55). Organ cultures of human embryonic trachea, while a sensitive culture system, are not practical for diagnostic laboratories. Although two strains have been adapted to growth in suckling mice (56), direct isolation in mice from respiratory tract specimens has not been reported.

SARS-CoV was isolated first in Vero E6 or fetal rhesus kidney cell lines with production of CPE (57–59). Vero E6 cells are now routinely used for its growth and also for plaque assays of infectivity (60). In addition, the virus has been adapted for growth in a number of other cell lines that express the ACE2 receptor. MERS-CoV was also initially isolated in Vero E6 cells, which remain a cell line used for growing and titrating this virus (11). There are reports that Caco-2 cells are more efficient for primary isolation (61).

Enteric coronaviruses have been very difficult to propagate *in vitro*, and most strains have only been detected by EM of negatively stained fecal preparations (62–65). Their morphology is sometimes different from that of other coronaviruses (66). On the other hand, several strains have been propagated in intestinal organ cultures (67, 68), and both

antigenic and biophysical studies have been performed (68, 69). Certain strains were related both to bovine diarrhea virus and to OC43 (69, 70). One strain, recovered from infants with outbreak-associated diarrhea and originally isolated in fetal intestinal organ culture, has been adapted to growth in a mouse macrophage line and a mosquito cell line and appears unrelated to other HCoVs or animal coronaviruses (71).

Inactivation by Physical and Chemical Agents

SARS-CoV and MERS-CoV remain viable for much longer than other HCoV or influenza when dried on surfaces (reviewed in 72). SARS-CoV remains viable up to 9 days in liquid suspension and 6 days in the dried state (73). SARS-CoV dried on smooth surfaces retains its viability for over 5 days at temperatures of 22–25°C and a relative humidity of 40–50% (i.e., typical air-conditioned environments), whereas virus viability is rapidly lost at higher temperatures and higher relative humidity (74). MERS-CoV also retains viability on surfaces for many days and is more stable at low-temperature/low-humidity conditions. Aerosolized MERS-CoV retains viability at low temperature and low humidity (75). MERS-CoV has been isolated from environmental surfaces of patient rooms and anterooms, such as benchtops, bed sheets, bedrails, intravenous fluid hangers, and X-ray devices, for many days (76), indicating the potential for fomite transmission.

Common disinfectants commonly used in hospital and laboratory settings are generally effective in inactivating SARS-CoV. Thermal inactivation at 56°C was highly effective in the absence of protein, reducing the virus titer to below detectability; however, the addition of 20% protein exerted a protective effect resulting in residual infectivity. If protein-containing solutions are to be inactivated, heat treatment at 60°C for at least 30 min must be used (73).

EPIDEMIOLOGY

Geographic Distribution

Antibodies to OC43, 229E, NL63, and HKU1 have been detected in human sera worldwide. Seropositivity appears in early childhood and increases rapidly with age (77–79). Similarly, virus RNA from endemic HCoVs has been detected in clinical specimens globally (80–82). Seroprevalence of HKU1 in adults appears to be lower than observed with other endemic HCoV (78). It should be noted that the type of serological assay used may affect estimates of seroprevalence (see Laboratory Diagnosis section, below).

As noted above, SARS emerged in Guangdong Province, China, in late 2002 and spread to 29 countries (8). However, the interruption of human-to-human transmission in July 2003 aborted the outbreak. The virus re-emerged to infect four other persons in contact with live game animals in December 2003 and January 2004, the virus infecting humans being similar to SARS-like viruses circulating in civets and other animals within these markets. These patients had mild disease and no secondary transmission was detected. The closure of these live game animal markets prevented further human zoonotic infections.

Three laboratory-associated infections have subsequently been reported, one of them leading to limited secondary transmission within the community (8, 83). SARS was unusual among respiratory viruses in that asymptomatic infection was uncommon (84). Thus, antibody to SARS-CoV is found only in those who have had clinical SARS, a small number of contacts who have been asymptomatically infected, and a fraction of individuals who work in these live-animal markets and have presumably been exposed to the precursor SARS-CoV-like virus (40). The continued presence of the SARS-like bat virus precursor in *Rhinolophus* bats implies that the re-emergence of SARS remains possible (39).

MERS-CoV still remains zoonotic in origin, all zoonotic infections so far being reported from the Arabian Peninsula or the Middle East region (85). Travel-associated cases have been reported in many other countries in Europe, Africa, and Asia, some of them leading to secondary transmission; the largest such outbreak outside of the Middle East affected 186 persons in the Republic of Korea in 2015 (85, 86). Although dromedary camels are infected endemically in North, East, and Central Africa (87), primary zoonotic infections have not been reported so far in Africa. It is unclear whether this reflects lack of recognition and diagnosis, differences in zoonotic potential of the virus, or other behavioral factors that affect transmission.

The geographic distribution of the gastrointestinal coronaviruses is less clearly delineated. CVLPs have been found in the stools of adults and children in both resource-rich and -poor countries, and it has been common to find them in equal frequencies in both healthy and sick persons.

Incidence and Prevalence

Endemic HCoVs (229E, OC43, NL63, HKU1)

The rate of coronavirus infection among adults with upper respiratory illness varies by respiratory seasons and year. In an early 6-year study of 229E infection among medical students, based solely on rises in neutralizing antibody, only 1% of acute respiratory illnesses in the period from 1964 to 1965 could be attributed to 229E; but from 1966 to 1967, the proportion was 35%, giving an overall average infection attack rate of 15% (88). The proportion of coronavirus-associated minor respiratory illnesses in a general population in Tecumseh, MI, during the same peak year was 34% (89), and a rate of 24% was found in Bethesda, MD (48). Serosurveys of OC43 infection in adults have shown similar proportions. During peak seasons, 25% (89) to 29% (77) of colds could be associated with OC43 infection; overall, 17% of individuals developed antibody rises each year.

In a serologic survey of OC43 infection in high-risk adult populations in Houston, TX, 8–9% of acute respiratory episodes in outpatient adults with underlying chronic obstructive pulmonary disease or asthma were attributable to OC43 infection (90). In England, a study of asthmatic adults with acute respiratory symptoms from 1990 to 1992 showed infection with OC43 or 229E in 16% (91). Serologically documented infections by 229E or OC43 occur at about one-half the frequency of rhinovirus infection and the same as, or somewhat greater than, that of influenza virus and respiratory syncytial virus (RSV) infections (92, 93). Among Finnish adults surveyed by serologic techniques over a 10-month period, 8.5% of colds were associated with infection with either 229E- or OC43-related strains compared to 52.5% rhinoviruses detected by reverse transcriptase PCR (RT-PCR) (94). There are few longitudinal seroepidemiological studies with HCoVs NL63 or HKU1. In a small cohort (n=13) of newborn babies followed serologically for 18 months using an NL63 and 229E nucleocapsid protein-based enzyme-linked immunosorbent assay (ELISA), all of the babies had maternally acquired antibodies at birth. After clearance of maternal antibody, 7 and 2 of these

13 children became NL63 and 229E seropositive, respectively, during the 18 months of follow-up (95).

Using RT-PCR for detection of 229E and OC43, two community studies of acute respiratory illness have been performed in patients cared for by general practitioners in The Netherlands (96, 97). In adults 60 years or older, during a single respiratory season, these two coronaviruses were found in 17% of 107 elderly subjects with acute respiratory disease, in contrast to only 2% of controls. In the same cohort, 32% of episodes were associated with rhinovirus infection (2% of controls), and 7% were associated with influenza infection (0% of controls) (96). In the second study, subjects of all ages (mean, 35 years) were sampled over 3 years. In contrast to the findings in the exclusively elderly, in this population coronavirus infection was not significantly associated with illness, being found in 6 (3.6%) of 166 influenza-like illnesses, 29 (7.7%) of 376 other respiratory illness, and 21 (3.9%) of 541 controls (97). There have been no systematic, adequately controlled studies of either NL63 or HKU1 in adults or children with outpatient respiratory illness.

Particularly in young children, coronavirus RNA is frequently found in respiratory samples from asymptomatic as well as symptomatic individuals (97–99). Also, multiple viruses may be detected in the same clinical specimen, such that it is often difficult to attribute illness, especially of the lower respiratory tract, in an individual patient. The level of confidence of causality of HCoV for lower respiratory tract infections is enhanced by including an age-matched control group or by detecting the virus in specimens such as bronchoalveolar lavage (BAL), lung biopsy, or autopsy specimens, although the latter are rarely available. Concomitant seroconversion also enhances confidence that the detected virus is of clinical relevance. Infection rates in six studies of outpatient or hospitalized patients with acute respiratory tract disease are summarized in Table 1 (81, 100–104). In outpatient or hospitalized patients with acute respiratory tract disease, in which 229E, OC43, NL63, and HKU1 were sought by RT-PCR, there was evidence of HCoV RNA in 1.7–7.6% of patients, with varying proportions of the four HCoVs being detected. Coinfections with other respiratory viruses were detected in about one-quarter of the HCoV infections, similar rates of coinfections being found for each of the four HCoVs. In a multiple-year study of acute respiratory illness of all ages in Yamagata, Japan, HCoVs were detected in 7.6% of infections overall, all four endemic HCoVs being detected. There was marked winter seasonality, and monthly detection rates of NL63 exceeded 10% in 3 months, with peak monthly detection rates being as high as 28.5% (81). In a study of children hospitalized with acute respiratory symptoms or fever, HCoV-associated hospitalization per 10,000 population was 10.2 [95% confidence interval (CI) 4.3, 17.6], 4.2 (95% CI 1.9, 6.9), and 0 (95% CI 0, 3.7) in children aged <6 months, 6–23 months, and 24–59 months, respectively (105). In an outpatient-based study where all nontrauma acute illnesses were investigated, acute respiratory symptoms were the most common presenting symptom, but diarrhea was noted as a presenting symptom in some (80). Of those patients with HCoV being the only virus detected, 7.7%, 9.0%, and 9.1% of the patients with NL63, 229E, and OC43 required hospital admission. Peaks of activity with one virus often were associated with subsequent periods (years) of low virus activity (80, 81).

Enteric coronaviruses or CVLPs have been most frequently associated with gastrointestinal disease in neonates and infants less than 12 months of age. Particles have been found in the stools of adults with acquired immunodeficiency syndrome (AIDS), in some studies more frequently in the presence of diarrhea than in its absence (106). Asymptomatic shedding is common, and particles are apparently shed for prolonged periods (65, 107–109).

SARS-CoV and MERS-CoV

During the period of the SARS outbreak (from November 2002 to July 2003), 8,096 confirmed cases were reported from 29 countries or administrative regions across five continents, 774 of these being fatal. After the end of the outbreak in July 2003, four laboratory-acquired infections were reported in Singapore, Taiwan, and Beijing in 2003 and 2004, one of these leading to limited community transmission, which was again controlled by public health measures (83). Another four instances of new zoonotic transmission occurred in December 2003 to January 2004 in live-animal markets, in Guangdong Province, China, causing mild disease, and did not result in detectable human-to-human transmission (110).

As of early January 2016, 1,626 cases of confirmed MERS had been reported from 26 countries in Asia, Africa, Europe, and North America, 586 of these being fatal (111). Primary zoonotic infections have so far only been reported from the Arabian Peninsula and the Middle East, with travel associated cases being reported from other countries.

Seasonality

In temperate regions, OC43, 229E, and, on the basis of more limited data, also NL63 and HKU1 appear to have peak incidence in the winter and early spring (55, 81, 112, 113), but seasonality may be more variable in tropical and semitemperate regions (100, 114). Two or three yearly cycles have been observed with each coronavirus, possibly a result of population immunity. Unlike seasonal influenza, in which a given subtype and strain may dominate in multiple countries in any given year, coronavirus outbreaks are more localized geographically (112).

Reported cases of MERS appear to show marked seasonal peaks in the spring-summer period (85). Some of these peaks are due to large outbreaks within hospitals, and longer-term follow-up would be required to establish if such a seasonal pattern does exist. Zoonotic MERS-CoV infections may increase in the spring, following an increase in virus activity in camels associated with the camel calving season, which occurs in the winter and early spring. Higher rates of detection of MERS-CoV are found in camel calves compared with adults (115).

On the basis of the limited data currently available, enteric coronaviruses appear to have little or no seasonality (116).

Transmission

Endemic HCoVs

Both 229E and OC43, as well as several less-well-characterized strains of coronaviruses (B814, LP, EVS, OC16, OC37, OC38, OC44, and OC48) were transmitted by intranasal inoculation to adult volunteers in the Common Cold Research Unit, Salisbury, UK, and all produced clinical upper respiratory illness (117). Presumably, the respiratory route is the primary mode of infection with these viruses, although the details of their spread have not been studied in a field setting. After infection of adult volunteers, virus was shed beginning 48 hr after inoculation, at about the time

TABLE 1 Summary of selected epidemiological studies on the prevalence of respiratory coronavirus infection in outpatient and inpatient populations. Data on other respiratory viruses are shown for comparison, where available.

Parameter	Description in reference					
	McIntosh et al. (102)	Esposito et al. (103)	Matoba et al. (81)	Lau et al. (100)	Choi et al. (101)	Kuypers et al. (104)
Population sampled	Inpatients, children	Outpatients, children	Outpatients, all ages	Inpatients, all ages	Inpatients, children	Inpatients and ER, children
Location	Chicago, IL	Milan, Italy	Yamagata, Japan	Hong Kong	Seoul, South Korea	Seattle, WA
Patient characteristics	Bronchiolitis, pneumonia	All acute nontrauma admissions	Acute respiratory illness	Acute respiratory illness	Acute respiratory illness	Acute respiratory illness
No. of patients	380	2,060	4,324	4,181	515	1,061
No. of respiratory seasons	4	1	4	1	5	1
Method for HCoV detection	Serology	RT-PCR	RT-PCR	RT-PCR	RT-PCR	RT-PCR
Coronavirus(es) sought	229E, OC43	229E, OC43, NL63, HKU1	229E, OC43, NL63, HKU1	229E, OC43, NL63, HKU1	229E, OC43, NL63	229E, OC43, NL63, HKU1
All respiratory viruses (% positive)	55.0	41	61.9	41.2	60.6	NR
RSV (%)	27.9	8.3	NR	10	23.7	23
Rhinovirus (%)	NT	6.3	NR	NT	5.8	NT
Influenza viruses (%)	4.0	11.4	NR	15.9	6.4	12
Parainfluenza viruses (%)	27.5	1.4	NR	5.3	8.0	9
Human metapneumovirus (%)	NT	2.3	NR	2.8	4.7	7
Adenovirus (%)	6.8	6.6	NR	5.0	6.8	13
Enteroviruses (%)	NT	NT	NR	NT	NT	NT
Human bocavirus (%)	NT	NT	NR	NT	11.3	NT
Coronaviruses (%)	7.9	3.8	7.6	2.1	1.7	6.3
229E%		2.0	0.9	0.1		
NL63%		1.0	3.1	0.4		
OC43%		0.8	1.8	1.3		
HKU1%		0	1.9	0.3		
Noncoronavirus coinfection rate (%)	NR	NR	NR	NR	11.5	NT
Coronavirus coinfection rate (%)	NR	28	24.4	NR	NR	NT

HCoV, human coronavirus; RT-PCR, reverse transcriptase PCR; RSV, respiratory syncytial virus; NR, not reported; NT, not tested.

symptoms began, and shedding continued for five days (118). Presumably, infected subjects are themselves infectious during this time. Comparable studies have not been carried out with NL63 or HKU1. Detailed information is lacking on the mode of spread of the endemic respiratory HCoV infections; i.e., the relative importance of small versus large droplets and aerosols or fomite transmission. In community-based settings, there is evidence of clustering of transmission within family groups (112).

SARS-CoV

The community transmission patterns for SARS-CoV are very different from endemic HCoVs. Although the majority of cases did not transmit infection at all, a few were responsible for explosive outbreaks, the so-called "superspreading incidents" (119). In a number of these instances, it is the overall epidemiological context rather than the nature of the individual index patient that was crucial to such super-spreading events. An unusual transmission event occurred in Amoy Gardens, an apartment complex in Hong Kong, where over 300 people acquired infection within a few days from one index case who had diarrhea as well as mild respiratory illness at that time. It is believed that a faulty sewage system led to aerosolization of infectious fecal matter, which then spread by the airborne route to affect many other residents in this housing complex (120).

The number of secondary cases produced by a single case was estimated to be 2.2 to 3.7, similar to what is estimated for pandemic influenza (121). The success of public health measures in interrupting transmission is that, unlike with many other respiratory viral infections, it predominantly took place later in the illness, usually after day 5 of symp-

toms. This provided a window of opportunity for case detection and isolation prior to maximal transmissibility, allowing public health measures to interrupt transmission in the community (122). In addition, there was extraordinary cooperation and communication between countries and public health workers to control spread. It is interesting to speculate whether SARS-CoV might have become an endemic respiratory infection if not for the determined international global public health efforts implemented in 2003.

MERS-CoV

MERS-CoV infection in humans has been initiated by zoonotic transmission events, camels being the likely source of such infection (36). Infection in camels is mild, causing a "common cold"–like illness, especially in juveniles (123). The virus is shed primarily from the nose of infected camels, with virus being shed for a few weeks. Virus is less often detected in the feces. Although virus RNA was detected in camel milk, it has so far not been cultured from milk (124). Given that milk is usually rich in maternally transferred antibody to MERS-CoV, it is unclear if such contaminated milk is infectious. However, given the local practice of drinking fresh, unboiled camel milk, this must be considered as a potential route of transmission. Camels become reinfected, resulting in a high prevalence of MERS-CoV shedding in situations where animals from different origins are mixed, such as camel abattoirs. Thus, virus shedding is common in such settings and implies constant exposure of humans to infected animals although disease does not appear to be common.

A large population-based seroepidemiological study of apparently healthy persons older than 15 years of age in Saudi Arabia revealed an overall seroprevalence of 0.2%, increasing to 3.6% in abattoir workers routinely exposed to dromedary camels, implying that there have been many more infections taking place than has been recognized from case diagnosis (125). Whereas nosocomial transmission accounts for most of the transmission occurring between humans (see next section), transmission does occur within family settings (126). There is evidence of asymptomatic infection of humans infected zoonotically (127) as well as nosocomially (128), and transmission possibly occurs from such asymptomatic or mild infections (129). Thus, a likely scenario is that most zoonotic transmission is asymptomatic or mild and unrecognized. Such infections may sometimes lead to transmission to other humans, where infection may be detected only in those in whom severe clinical disease occurs, for example, because of being older in age or having underlying comorbidities. In such epidemiological settings, it may be difficult to identify the initial zoonotic infection.

Nosocomial Transmission

As with other respiratory viruses, nosocomial transmission of coronaviruses is problematic. One outbreak of respiratory endemic HCoV in a neonatal intensive care unit caused 10 infections, all associated with symptoms of generalized acute illness, among 40 premature infants (130). NL63 infection in hospitalized children has also led to an outbreak in a neonatal intensive care unit (131). Occurrence of infections in closed populations, such as military or children's institutions, have been reported (112). However, the most dramatic examples of nosocomial transmission of coronaviruses occurred with SARS and MERS.

Both SARS and MERS have been merciless in exploiting lapses in infection control measures within healthcare settings, particularly within modern hospital settings. During the SARS outbreak in 2003, 21% of cases were in healthcare workers, often with severe consequences. The virus spread readily in the hospital environment, particularly early in the epidemic of 2002–2003, when recognition of the disease was poor, confirmatory diagnosis was lacking, and appropriate precautions were not being taken (132). The enforcement of droplet and contact precautions was strongly associated with protection (133). However, in some instances when aerosol-generating procedures were used (e.g., nebulizers, intubation, and high-flow oxygen therapy), transmission also occurred via small-particle aerosols. The unusual stability of the virus also likely predisposed it to spread via direct or indirect contact (see above). Risk factors associated with SARS outbreaks in hospital wards were narrow space between beds, lack of availability of washing or changing facilities for staff, performance of resuscitation in the ward, and the use of oxygen therapy or bilevel positive-airway-pressure ventilation (134).

With MERS-CoV, chains of transmission have led to large outbreaks within healthcare facilities, some involving over 100 persons and often involving healthcare workers (86, 135). Some outbreaks have involved multiple healthcare facilities and have continued for months (136). Outbreaks of MERS-CoV have been associated with failures in consistent application of patient triage and infection control precautions; failure in early recognition, diagnosis, and isolation; crowded emergency rooms; and healthcare workers continuing to work while infected in the early stages with mild illness or asymptomatically (129, 136). An outbreak in the Republic of Korea initiated by a returning traveler led to 185 other confirmed infections in 16 hospitals. Some 44.1% of the cases were patients exposed in hospitals, 32.8% were family or paid caregivers, and 13.4% were healthcare personnel (86). Although 91% of cases did not lead to secondary transmission, five "superspreaders," all of whom had pneumonia at initial presentation and came into contact with large numbers of people prior to diagnosis, led to 83% of the cases. In the Korean outbreak, the practice of seeking healthcare at multiple healthcare facilities, of having paid caregivers within hospitals, and frequent interhospital transfers were additional factors that appear to have contributed to spread. Heightened awareness, infection control measures, patient isolation, and quarantine and surveillance of known contacts have so far been able to contain such outbreaks, as illustrated in Saudi Arabia and Korea.

Risk Factors

Endemic HCoVs

In contrast to other respiratory viruses, infection attack rates of HCoV in young adults are not much lower than those in children (89). There is no consistent sex difference in susceptibility, although an excess of females has been noted in some community-based studies (112). Increased susceptibility of females to experimental challenge has been noted (137). HCoV infections (229E, OC43, HKU1, NL63) have been associated with exacerbation of chronic obstructive respiratory illnesses, but it is unclear if this reflects increased susceptibility to infection or more severe illness following infection (138). Susceptibility to experimental infection by 229E (as well as to rhinoviruses) is increased by psychological stress (139). Atopy [as assessed by detectable total immunoglobulin E (IgE) in nasal secretions or higher serum IgE levels] is associated with increased clinical severity following

experimental 229E infection (140). Immunocompromise appears to be associated with severe HCoV disease, although whether because of increased susceptibility, severity, or both is unclear (141, 142).

SARS and MERS

Increasing age has been associated with increased severity of disease for both SARS and MERS. In Hong Kong, mortality rates in patients with SARS aged 0–24, 25–44, 45–64, or >65 years was 0%, 6%, 15%, and 52%, respectively (reviewed in 8). Severity of MERS also increased with age, but this may reflect the presence of comorbidities as well as age. Of reported cases, 76% of patients with MERS had underlying comorbid conditions (obesity, diabetes, cardiac disease, lung disease, immune-compromised) compared to 10–30% of patients with SARS (143). In the case of MERS, it is likely that the older age of reported cases reflects the fact that younger patients have milder disease and remain undiagnosed. Genetic polymorphisms associated with low serum levels of mannose-binding lectin were associated with increased susceptibility to SARS (144). Genetic factors associated with the risk of MERS remain to be investigated.

The risk of zoonotic MERS is associated with exposure of the patient or family members to dromedary camels (37).

PATHOGENESIS IN HUMANS

Patterns of Virus Replication

In volunteers infected experimentally with 229E and OC43 HCoVs, infection was detectable in the respiratory tract from around the time of symptom onset for about five days (145, 146). During acute infection, all six HCoVs replicate primarily within the respiratory tract (see section on Pathogenesis, below). HKU1 has been detected in the feces of patients with gastroenteritis, but its contribution to these symptoms is still unclear (147).

Viral load of SARS-CoV RNA, as quantitated by RT-PCR in the upper respiratory tract (nasopharyngeal aspirates), was low in the first 4–5 days of illness but peaked in the second week, corresponding to the time at which patients were maximally infectious (148). Virus RNA was detectable in the respiratory secretions, feces, and, at lower frequency, in urine, for 3–5 weeks, even though transmission was uncommon after the third week of illness (149). SARS-CoV replicated in the gastrointestinal tract, as demonstrated by EM evidence of viral replication in intestinal epithelium (apical enterocytes) in intestinal biopsies and autopsy tissues (150). Virus RNA was routinely detected in serum and urine, although it was not certain whether this reflected spillover of virus replicating in the respiratory and gastrointestinal tracts. Viral load in nasopharyngeal aspirates and in serum from day 10 to day 15 after onset of symptoms was associated with oxygen desaturation, mechanical ventilation, diarrhea, hepatic dysfunction, and death, whereas stool viral load was associated with diarrhea (151). Furthermore, higher initial viral load was independently associated with worse prognosis in SARS (152).

MERS-CoV RNA has been detected in respiratory specimens, with higher viral load being found in the lower respiratory tract (endotracheal aspirates, sputum) than in upper respiratory specimens (reviewed in 153). The viral load is also higher in fatal cases than in those who survive (154, 155). Although virus RNA has also been detected in the serum, feces, and urine, there is so far no direct evidence that there is active replication at these sites, although such replication cannot be excluded. Virus RNA can be detected for over 30 days after the onset of illness (156), but the probability of isolating virus from clinical specimens decreased with time after hospital admission and with decreasing viral load (61).

Pathology

A histopathological study described the nasal mucosa of a young girl with chronic rhinitis and bronchitis who showed the typical EM changes of a coronavirus infection (157). Brush biopsy specimens showed morphologically typical coronavirus particles in large numbers in cytoplasmic vesicles and the Golgi apparatus of ciliated epithelial cells (and not in goblet cells). Interestingly, the infected cells appeared not to show signs of cell death and appeared to have intact synthetic activity. On the other hand, degenerative changes affecting the cilia and loss of cilia were seen, likely indicating decreased ciliary function. It is interesting that EM of SARS-CoV infection of the human gastrointestinal tract seems to reveal a similar pattern, with viral replication and budding occurring with minimal CPE (150, 158).

In SARS, type 1 and type 2 pneumocytes are the key target cells for the virus, and pulmonary histolopathology varied according to the duration of illness virus (159–161). Cases of 10 or fewer days' duration demonstrated acute-phase diffuse alveolar damage (DAD), airspace edema, and bronchiolar fibrin. Cases of more than 10 days' duration exhibited organizing-phase DAD, type II pneumocyte hyperplasia, squamous metaplasia, multinucleated giant cells, and acute bronchopneumonia. Multinucleated cells were observed to be pneumocytes or macrophages (159, 160). Immunohistochemistry and *in situ* hybridization studies showed evidence of SARS-CoV infection in the alveolar epithelium and alveolar macrophages in lungs of patients dying within the first 2 weeks of illness, but not at later times after disease onset. Although the virus spreads to other organs (e.g., the gastrointestinal tract) and symptoms of diarrhea were common, disease severity and fatality were due to the respiratory pathology. Pathological studies of MERS are limited. In a 45-year-old man who died on day 8 after onset of illness, the main histopathologic finding in the lungs was diffuse alveolar damage. Pneumocytes and epithelial syncytial cells showed MERS-CoV antigen by immunohistochemistry. There was no evidence of viral antigen in extrapulmonary sites (162).

Pathogenesis

Endemic HCoVs

Little is known of the pathogenesis of the endemic HCoVs. The symptom profile is very similar to that of rhinovirus-induced colds, and likely comparable mechanisms leading to upper respiratory tract illness apply (see Chapter 47).

SARS-CoV

The pathology and viral tropism of SARS-CoV have been discussed above (161). Whether and to what extent immunopathology contributes to the disease process is still unresolved (8). Proinflammatory cytokines, such as interleukin 1 (IL-1), IL-6, and IL-12, and chemokines, such as IL-8, chemokine (C-C motif) ligand 2 (CCL-2), and C-X-C motif chemokine 10 (CXCL10), have been found to be elevated in plasma of patients with SARS, but it is not clear whether they drive pathogenesis or are a reflection of virus-induced cell pathology. *In vitro* studies suggest that poor interferon (IFN) induction and signaling occur in SARS-

CoV-infected macrophages and monocyte-derived dendritic cells (reviewed in 8, 163, 164). However, plasmacytoid dendritic cells (pDCs) respond to SARS-CoV infection with potent type I interferon responses (165). Transcriptomic studies of peripheral blood leukocytes from SARS-CoV-infected patients early in the disease process revealed high IFN-alpha, IFN-gamma, IFN-stimulated chemokine, and IFN-stimulated gene (ISG) expression. SARS patients who recovered then expressed adaptive immune genes, whereas those with poor clinical outcomes showed deviated ISG expression, immunoglobulin gene expression levels, persistent plasma chemokine levels, and deficient anti-SARS spike antibody production, suggesting a malfunction of the switch from innate immunity to adaptive immunity (166).

Aged macaques infected experimentally with SARS-CoV developed more severe pathology than young adult animals (see above). Although levels of viral replication were similar in both age groups, older animals had pronounced activation of genes associated with inflammation such as nuclear factor-kappaB (NF-kappaB)-related pathways while expression of IFN-beta was reduced. Therapeutic treatment of SARS-CoV-infected aged macaques with type I IFN reduces pathology and diminishes pro-inflammatory gene expression, including IL-8 levels, without affecting virus replication in the lungs (167).

MERS-CoV

The pathogenesis of MERS is less well studied, and only limited data exist for humans infected with MERS-CoV. Experimental infection of *ex vivo* cultures of human bronchus and lung demonstrate that nonciliated epithelial cells in the bronchus, type 1 and type 2 pneumocytes in the lung, and lung vascular endothelium are infected by the virus, reflecting the distribution of the DPP4 receptor, whereas HCoV 229E did not replicate in human bronchus or lung (168). MERS-CoV infects and replicates in the alveolar epithelial cell line A549, but there are no detectable type I or III IFN responses (168). Human B cells, M1 macrophages, M2 macrophages, and monocyte-derived dendritic cells did not secrete type I or type III IFNs upon inoculation with MERS-CoV *in vitro*. In contrast, human pDCs secreted large amounts of type I and III IFNs upon contact with MERS-CoV (169). MERS-CoV abortively infects CD4 and CD8 T cells, leading to their apoptosis and possibly explaining the lymphopenia observed in MERS (170). In BAL cells of two MERS patients collected in the first week of illness, the patient with a fatal outcome had poor induction of retinoic acid-inducible gene 1 (RIG-1), melanoma differentiation associated protein 5 (MDA-5), interferon regulatory factor 3 (IRF3), IRF7, and type I IFN, whereas the patient who survived had strong type I IFN responses (171).

Immune Responses

Endemic HCoVs

Serum antibody to the major structural antigens of the virus (primarily to the S protein but also to the M and N proteins) is made in adult volunteers in response to inoculation and infection with HCoV 229E and OC43 (172). Antibody titers, as measured by ELISA, rise significantly in volunteers who shed virus (173). In experimental human challenge studies with HCoV 229E, both circulating and mucosal-specific antibodies are associated with protection from infection and disease, but only specific IgA antibodies appear to shorten the period of virus shedding. Total protein in nasal washings also appeared to protect against infection, indicating that other locally produced proteins, not yet identified, may be associated with resistance (137). Volunteer studies with 229E and 229E-like strains suggest that symptomatic reinfection after a period of 1 year is possible. It is not clear, however, whether this is due to waning immunity or to slight differences in the antigenicities of different virus strains (174). Sequencing of several variants of 229E and OC43 has revealed somewhat contradictory data regarding the genetic diversity of the S protein over time and location, but with consensus regarding the lack of evidence for recombination events (175, 176). Although T cell immunity has been well studied in mice, there are limited data on T-cell responses following human coronavirus infections (reviewed in 177).

SARS-CoV

The S protein is the predominant target of neutralizing humoral immunity, and the major antibody neutralizing epitopes are in the region from residues 441 to 700 of the S protein of SARS-CoV (8). Neutralizing antibody titers were shown to rise in the second week of the illness, peak between week 5 and week 8 after onset, and decline thereafter, with a half-life of 6.4 weeks (178). All patients who survived >28 days of illness became seropositive. By 36 months after illness, SARS-CoV IgG and neutralizing antibody were undetectable in 25.8% and 16.1% of patients, respectively (179). Contradictory findings have been reported on the association between early antibody responses and disease severity (178, 179), but the use of corticosteroid therapy may have confounded these associations. In patients that have recovered from SARS, T-cell responses have been demonstrated to both the N and S viral proteins in convalescence, and memory T-cell responses have been detectable even 6 years after infection (reviewed in 177).

MERS-CoV

MERS-CoV infection is associated with robust neutralization and MERS-S1 ELISA antibody responses in most survivors, typically within 3 weeks of the onset of illness, but a few patients have marginal or undetectable antibody responses even after 4 weeks of illness (154, 180). There is yet little information on the longer-term kinetics of antibody responses in MERS-CoV infections. Serum antibody titers correlate inversely with lower respiratory tract viral RNA loads, but virus RNA may remain detectable for weeks despite development of neutralizing antibody responses (154). No systematic studies of T-cell responses in patients with MERS have been reported. The neutralizing antibody responses are directed primarily at the receptor-binding domain of the spike (S1 domain) protein; however, a minor component of neutralizing antibody response binds to other parts of the S1 and S2 domains of the spike protein (181). Although there is genetic diversity in MERS-CoV, all variants appear to be one serotype with efficient cross-neutralization between strains (61, 182).

CLINICAL MANIFESTATIONS

Endemic Human Respiratory Coronaviruses

Association of the HCoV strains 229E and OC43 with human disease has been established conclusively by infecting volunteers intranasally with cultured virus and documenting "common cold"–like symptoms correlated with evidence of viral replication in the upper respiratory tract (117, 145, 146, 174, 183). Only 10–30% of inoculated

volunteers developed symptoms, indicating that most HCoV 229E and OC43 infections are asymptomatic in healthy adults. The incubation period of 229E and OC43 infection in volunteers averages 3 days with a range of 2–5 days (145). The peak of respiratory symptoms is not reached until 3 or 4 days after inoculation. The clinical illness consists of general malaise, headache, nasal discharge, sneezing, and a sore throat, symptoms that last for 6–7 days. Cough is seen in about 25% of the subjects and fever only in approximately 10%. The features of coronavirus URTI cannot be differentiated from those produced by rhinoviruses, but fever, chills, and myalgia are less prominent than with influenza. Overall, the character and severity of HCoV illness are somewhat less severe than those of influenza virus and RSV, rarely leading to hospitalization (92, 93, 95). In a study of children with upper and lower respiratory illness that did use a control group for comparison, an association of PCR detection of HCoV 229E and OC43 with upper and lower respiratory disease has been demonstrated (184). Similarly, NL63 has been found preferentially in children hospitalized with croup, even more commonly than parainfluenza viruses, which have been traditionally associated with this disease (101, 185, 186).

A study of 418 patients (mean age 49 years) with community-acquired pneumonia (CAP) in Hong Kong over a 1-year period (2003–2004) yielded HKU1 in 10 (2.4%), 9 of them adults (187), although no control group was studied. The median age of the HKU1-positive patients was 71.5 years (range, 13–96 years); eight of them had underlying comorbidities and two had fatal outcomes. Clinically, these patients were not distinguishable from age-matched CAP patients who were HKU1 negative. Coronavirus infection of marine recruits has been associated with pneumonia and pleural reaction in about 33% (188). A 20-month survey testing all four HCoV types in 540 BAL samples from 279 hospitalized adults identified HCoVs in 29 (5.4%) samples, one-third of all respiratory viruses detected (189). HCoV OC43 was identified in 12, 229E in 7, NL63 in 6, and HKU1 in 4 specimens. Most of the HCoV-positive patients had clinical and radiological evidence of pneumonia and were immune suppressed or were lung transplant recipients.

As with other respiratory viruses, prolonged shedding of virus with or without severe disease can occur in immunocompromised patients (141, 142, 190). Endemic HCoVs are detected in hematopoietic stem cell transplant recipients; the median duration of viral shedding is 3 weeks, but prolonged shedding of at least 3 months can occur. Infection can be asymptomatic or associated with upper respiratory tract symptoms. Progression to lower respiratory tract involvement may occur rarely (191, 192).

Acute lower respiratory tract viral infections in patients after lung transplantation are associated with respiratory viruses in 66%, with coronaviruses (OC43, 229E, and NL63) being the second most common behind rhinoviruses (193). A highly significant association exists between viral infection and decline in 1-sec forced expiratory volume (FEV-1), acute rejection, and likely development of bronchiolitis obliterans syndrome.

In young children with a history of asthma, acute exacerbations were seen during infection by OC43 and 229E (194), although recent studies using PCR have shown that rhinoviruses are by far the most important cause of triggering acute wheezing in children with underlying asthma (195). In adults with chronic pulmonary disease or asthma, several serologic studies have shown significant association between coronavirus infection and acute exacerbations of respiratory symptoms (90, 91, 96, 196, 197). Infection in the elderly, particularly in those with underlying cardiopulmonary disease, is commonly associated with lower respiratory tract symptoms, although these rarely lead either to hospitalization or to death (92, 93).

The role of respiratory coronaviruses in otitis media has been elucidated by detecting viral RNA in both nasal secretions and middle ear fluids. Among 92 children with acute otitis media, coronavirus sequences were found in 16 children (17%), with 14 children harboring the virus in the nasopharynx and 7 harboring it in the middle ear fluid (198). This incidence was lower than for RSV (28%) and rhinovirus (35%). Coronaviruses were less frequently found in middle ear effusions at the time of tube placement (3 of 100) (199).

Overall, 229E, OC43, NL63, and HKU1 are able to cause upper respiratory tract infections, croup, otitis media, as well as lower respiratory tract infections, the latter being more commonly seen in children, the elderly, or those with comorbidities (80).

More controversially, there is also evidence for the presence and persistence of 229E and OC43 virus RNA in the central nervous system (CNS) in conjunction with chronic neurologic syndromes, particularly multiple sclerosis and acute demyelinating encephalomyelitis (200–203). Human "respiratory" coronaviruses are sometimes capable of entering the CNS. However, assignment of a pathogenic role in demyelinating diseases of humans, so well demonstrated in the murine model, must, however, await further studies.

SARS-CoV

The incubation period for SARS-CoV has been estimated to average 4–6 days, with a range of 1–14 days (121, 122). SARS begins with acute onset of fever, myalgia, malaise, and chills and then progresses to cough. Upper respiratory symptoms of rhinorrhea and sore throat are uncommon. Dyspnea and tachypnea develop later in the illness; at this stage, individuals often have scattered ground-glass peripheral lung infiltrates. Over the course of the next several days, they either improve gradually or worsen with increasing oxygen requirements; severe cases progress to acute respiratory distress syndrome (ARDS). A watery diarrhea occurs in some patients, usually associated with clinical deterioration, mainly in the second week of illness. Other extrapulmonary manifestations include hepatic dysfunction and CNS manifestations. Overall mortality is between 9% and 12%, mortality progressively increasing with age (reviewed in 8, 204, 205).

Chest radiographic abnormalities were present in 60–100% of patients, depending on duration of illness, and typically encompass ground-glass opacities or focal consolidation over the periphery and subpleural regions of the lower zones of the lung. Bilateral involvement and shifting opacities are common. High-resolution computed tomography (CT) scanning reveals abnormalities, even in those with initially normal chest radiography.

Laboratory abnormalities include leukopenia (particularly lymphopenia in severe cases) and elevated transaminase levels. In addition to age, the presence of comorbidities, more extensive lung involvement, high neutrophil counts, low CD4 and CD8 counts, and increased lactic dehydrogenase levels are predictors of a poor prognosis. High viral loads in nasopharynx and serum early in the illness and between days 10 and 15 of illness are independent predictors of a poor outcome.

Atypical (sometimes afebrile) presentations can occur in the elderly or immunocompromised patients, leading to delayed recognition, sometimes resulting in nosocomial transmission.

Three months after hospital discharge, patients convalescing from SARS have detectable defects in pulmonary function, but the impairment is mild in almost all cases. Many patients have reduced exercise capacity, not accounted for by impairment of pulmonary function (206).

MERS-CoV

The median incubation period for MERS is estimated to be approximately 5.5–6.5 days with a maximum 14-day incubation period used for contact tracing and management (85, 86). Clinical features of MERS range from asymptomatic infection to severe pneumonia, often leading to ARDS and death (reviewed in 143, 207–209). The range of illness is broadly similar to that of SARS, but the frequencies of subclinical and mild illness are much higher. Fever (98%), chills or rigors (87%), cough (dry or productive) (83%), and shortness of breath (72%) are common presenting symptoms; diarrhea or vomiting has been reported by around one-third of the patients, whereas sore throat (14%) and rhinorrhea (6%) are uncommon. Common chest radiographic abnormalities include bilateral hilar infiltrates, unilateral or bilateral patchy infiltrates, segmented or lobar opacities, and ground-glass opacities, with the lower lobes being generally more affected than the upper lobes, early in the illness. Small pleural effusions may sometimes be seen. Lymphopenia, thrombocytopenia, and high lactate dehydrogenase levels are seen in around one-third to one-half of patients.

Because mild and asymptomatic infections are more common in MERS, especially in younger persons such as healthcare workers, the case descriptions and case fatality ratios are likely biased by case ascertainment skewed to more severely ill patients. Patients reported with MERS have been older than those with SARS, and the overall disease progression of disease from onset to pneumonia (often within the first week), time to requirement of ventilator support, and time to death appear to be more rapid in MERS. Factors predisposing to fatal outcome include age, underlying comorbidities, and viral load in nasopharyngeal specimens, and, possibly, detection of viral RNA in plasma (155, 210).

Enteric Coronaviruses

The clinical features of possible enteric infections with coronaviruses have not been clearly described. CVLPs have been detected in stools from healthy subjects as frequently as in stools from those with enteritis (64). On the other hand, studies of disease in neonates and infants in the first year of life have found statistically significant associations between CVLPs and illness, either mild and self-limited (65, 69) or severe, and, in some neonates, requiring surgical intervention (62). One study comparing rotavirus and CVLP-associated diarrhea in children found similar incidences of fever and vomiting, but stools from children excreting CVLPs more often were positive for occult blood (18% versus 0%) and mucoid (32% versus 8%) (109).

LABORATORY DIAGNOSIS

Viral Detection

As for other respiratory viral infections, specimen collection should be targeted to the anatomical site predominantly affected (see Chapter 15). In clinical syndromes involving the upper respiratory tract, nasopharyngeal swabs or aspirates are the specimen of choice, with throat swabs being an alternative. In illnesses where the disease largely involves the lower respiratory tract with minimal involvement of the upper respiratory tract (SARS, MERS), lower respiratory specimens, including BAL, endotracheal aspirates (if the patient is intubated), or sputum, are appropriate. Sputum is not routinely considered a specimen of choice for viral diagnosis, but the experience with MERS suggests that sputum provides a higher diagnostic yield than throat, nasal, or nasopharyngeal specimens (154). However, optimization of methods for RNA extraction from sputum is needed to avoid occasional false-negative results because of high levels of mucus. In the case of suspected SARS or MERS, stool, urine, and blood (serum or plasma) are also useful specimens, although they generally give a lower diagnostic yield than lower respiratory specimens (154).

Primary isolation of respiratory HCoVs in cell culture is difficult, and serial passage is frequently required (see above). Respiratory HCoVs can be detected by immunofluorescence of cells shed from the respiratory tract using commercially available reagents (130) or polyclonal (211) or monoclonal (141) reagents developed in individual laboratories. ELISA for coronavirus antigen in nasal swabs or secretions has limited sensitivity (212). ELISA and other antigen detection assays for detecting MERS-CoV in camel swabs have been reported, and more evaluation with human specimens is awaited (213, 214).

RT-PCR, either conventional or real-time, has become the diagnostic method of choice for detection of all HCoV strains. There have been attempts to develop sets of "pancoronavirus" primers and probes (131, 215), and such systems have been used with success (193). However, type-specific primers have greater sensitivity. With HKU1 viruses, there are three genotypes, and it is important that the primers chosen will detect all of these (216). With SARS, the small amount of virus present in all clinical samples obtained in the early phase of the illness has proven to be a diagnostic challenge, even with sensitive RT-PCR methods. The use of multiple specimens (including stool and blood) increases diagnostic yields in the first few days of SARS illness. Diagnostic algorithms for MERS typically involve screening with RT-PCR primers targeting the RNA upstream of the E gene (*upE*) and confirmation with primers targeting ORFs 1b or 1a (217, 218). A single negative result does not exclude a diagnosis of MERS, and repeated testing using lower respiratory specimens must be carried out. Many of the widely used commercially available multiplex assays for detection of endemic respiratory viruses do not detect MERS-CoV; thus, it is important to make a specific request for MERS-CoV diagnosis when clinically indicated.

Genotyping of MERS-CoV can be carried out by primers targeting a 615-base-pair-long region in the S2 domain of the spike gene (219). The full viral genome (or large parts of it) can be sequenced directly from clinical specimens, especially when there is high viral load, and viral genetic analysis can complement epidemiological studies in investigating outbreaks (e.g., within hospitals) (136). Isolation of virus in cell culture often leads to the introduction of amino acid substitutions associated with cell culture adaptation (220). Thus, virus sequence analysis for molecular epidemiology is best based on virus sequence derived directly from the clinical specimen.

The diagnosis of enteric coronavirus infection depends on finding the characteristic particles in stool samples

examined by EM. No culture, antigen detection, or nucleic acid amplification system was available by early 2016.

Serology

A number of serological assays have been used for detecting coronavirus infections. Historically, complement fixation (CF), hemagglutination inhibition, and virus neutralization tests were used for epidemiological studies and for diagnosis of 229E and OC43 infections. CF antibody responses appeared relatively short lasting and possibly broadly cross-reactive across the then known as well as unknown endemic HCoV, whereas neutralizing antibody was more type specific and lasted many years (112). Neutralization tests (neutralization of CPE or plaque reduction neutralization tests) are thus generally regarded as the most specific and the "gold-standard" serologic tests. ELISA assays and immunofluorescence (IF) tests have also been used.

In IF tests, IgM antibodies in response to SARS remained detectable for more than 6 months after the onset of illness. While SARS CoV infections induce an anamnestic IgG antibody response to the 229E and OC43 viruses, these cross-reactive antibodies remain of high avidity from early (the first month) postinfection. Thus, assays to detect low-avidity antibody may be useful for discriminating early from late antibody responses and also for distinguishing anamnestic cross-reactive antibody responses from primary specific responses (221). Line immunoassays based on nucleoprotein to HCoVs 229E, OC43, NL63, HKU1, and SARS-CoV have been evaluated. There is considerable cross-reactivity between the two betacoronaviruses OC43 and HKU1 and between the betacoronaviruses 229E and NL63 (222). There is, however, no cross-reactivity with SARS-CoV.

For the serodiagnosis of MERS, ELISA or IF assays based on whole virus or virus-infected cells may sometimes detect cross-reacting antibody elicited by other coronaviruses. The S1 region of the spike protein is more specific. ELISA assays to detect MERS-CoV S1 antibody have been developed and commercialized (223). Antibody arrays using the S1 region of multiple human coronaviruses (including MERS-CoV) spotted on a glass slide have also been developed (36). Any sera positive with ELISA, IF, or antibody array tests need to be confirmed by a neutralization test. Conventional neutralization tests with viruses such as SARS-CoV or MERS-CoV require biosafety level-3 (BSL-3) containment because they necessitate the use of live virus. A pseudoparticle neutralization test expressing the MERS-CoV spike protein on the surface of a replication-incompetent human immunodeficiency virus (HIV) backbone with a reporter gene has proved to correlate very well with the plaque reduction neutralization test results (182, 224). This pseudoparticle neutralization test does not require BSL-3 containment and can be performed in BSL-2 laboratories. Kinetics of immune responses of SARS and MERS are discussed in the section on Immune Responses (above).

PREVENTION

General

Because MERS can be acquired zoonotically, it is important to educate persons living within MERS-endemic areas and those traveling to such regions to avoid close contact with dromedary camels, drinking fresh camel milk, contact with other camel secretions, and consuming under-cooked camel meat. If contact with these animals becomes unavoidable, appropriate hygiene measures should be used as soon as possible. The virus is shed mainly from the camel's nose and upper respiratory tract; thus, it is particularly important to avoid contact with these areas. In patients with confirmed MERS, in addition to infection prevention and control measures in healthcare settings, tracing and quarantine (within their homes or in hospital) of close contacts is required (225).

Infection Control and Prevention

As with other respiratory viruses, infection prevention and control measures for coronaviruses include administrative controls to manage patient flow, with clinical triage of those with acute respiratory infections, prevention of overcrowding in waiting areas, spatial separation of at least 1 meter between patients, adequate environmental ventilation, appropriate hand hygiene, and rational use of personal protective equipment. For patients with acute respiratory infections, standard precautions should be supplemented by the use of a medical mask when in close contact (1 meter) of a patient or when entering the patient room. Consider the use of a mask on the patient to minimize spread of infectious droplets, especially if it becomes essential to move the patient outside of the room. When performing aerosol-generating procedures (e.g., tracheal intubation, tracheotomy, noninvasive ventilation, manual ventilation prior to intubation, etc.), additional precautions (fit-tested particulate respirator, eye protection, long-sleeved gowns, fluid-resistant aprons) are needed (225).

For MERS, specific enquiry needs to be made about travel to MERS-endemic regions, evidence of risk exposures (camel contact, visit to healthcare facility in endemic areas, contact with known MERS patients), contact with an ill returning traveler from affected countries, or work in a laboratory where MERS-CoV exposure may have taken place. When MERS is suspected, additional precautions include the placement of the patient in a single room, preferably with negative pressure ventilation; limiting the number of healthcare workers, caregivers, and family members accessing the patient; sending appropriate specimens for urgent laboratory diagnosis; the use of a particulate respirator, eye protection, gown, gloves, and hand hygiene immediately before and after patient contact or entering the patient area; and the use of disposable or dedicated equipment (stethoscopes, blood pressure cuffs, thermometers, etc.). The use of portable X-ray equipment and other diagnostic equipment, which can minimize movement and transport of patients out of the barrier nursing area, needs to be considered (225). These additional barrier nursing precautions should be in place for the duration of the symptomatic illness and continued for at least 24 hr after symptoms have resolved. Patient information, such as immune status and use of immune-suppressive medication (e.g., corticosteroids), also needs to be considered. MERS-CoV viral load in respiratory specimens would also be useful to monitor in this context. However, it is not clear if low-level MERS-CoV detection by RT-PCR late in the illness necessarily implies that the patient is infectious.

Vaccines

At present, there is no effective vaccine for any of the human coronaviruses. Antibodies to the S protein of coronaviruses are neutralizing, the critical epitopes being those in the receptor-binding domain. Research on vaccine development for SARS-CoV was initiated after the outbreak in 2003, but there was waning of research funding when no

further cases occurred. Animal studies on vaccine-induced protection, on adoptive transfer, and on T-cell depletion suggest that antibody is necessary and sufficient to confer protection against SARS. Subunit vaccines, whole-virus inactivated vaccines, vaccines that use various live-virus vectors, and DNA vaccines have been tested in various animal model systems, and many of these modalities have shown promise (226). Phase 1 studies with an inactivated SARS-CoV vaccine demonstrated safety and immunogenicity (227). However, the persistence of the antibody responses was not assessed, and, furthermore, volunteers were young and healthy (age range 21–40 years); thus, immunogenicity in older persons remains unclear. Antigenic diversity and lack of cross-neutralization between the human SARS-CoV used for vaccine development and precursor SARS-CoV-like viruses found in small mammals in live-game markets (e.g., civets) and bats, both of which are likely sources of any new SARS outbreak, pose a problem for vaccine development (228). However, some monoclonal antibodies cross-neutralize and cross-protect against both human and animal (palm civet) coronaviruses, but perhaps not those from bats, which is one source of potential SARS re-emergence (229). Vaccines for coronaviruses carry the risk that paradoxical disease enhancement may occur, as has been seen with vaccines for feline peritonitis virus (230). Although such effects were not seen with many of the vaccines studied, there were some examples of vaccine-elicited immunopathology. Mice, ferrets, and macaques immunized with some SARS vaccines led to Th2-mediated immunopathology in the lung upon SARS-CoV challenge, an effect that was more pronounced in vaccines containing only the N protein (231).

Vaccines against MERS-CoV are being developed, primarily based on generating immunity to the spike protein. All MERS-CoV strains tested so far form one serotype with cross-neutralization between strains (see above) (61, 182). Thus, antigenic diversity may not be a problem for vaccine development. Spike DNA and subunit protein vaccines have provided protection in experimental mouse and primate model studies (181). Recombinant vaccines expressing MERS-CoV spike protein in a vaccinia vector have provided protection from experimental challenge in mouse models and in dromedary camels (232). A key remaining question pertains to the duration of such immunity, because there is evidence the natural infection does not prevent reinfection in camels. MERS vaccines are expected to go into Phase 1 human clinical trials soon (e.g., NCT 02670187).

Chemoprophylaxis

A double-blind, placebo-controlled study of self-administered intranasal recombinant human IFN-alpha A given both before and after virus challenge reduced incidence of colds, the severity of symptoms and signs, and virus replication as compared with those given placebo (233). Protection by intranasal IFN against HCoVs has not been demonstrated under field conditions.

In experimental animal models, MERS-immune antibody can effectively prevent and treat MERS-CoV infections (234, 235). Hence this approach was used as a primary treatment for a patient with MERS-CoV infection and as prophylaxis for his spouse, although it was not possible to assess efficacy in this anecdotal setting (236). Passive immunotherapy with polyclonal MERS-CoV immune serum or humanized MERS-CoV neutralizing monoclonal antibodies is effective in prophylaxis in experimental models of MERS (237). Thus, this potential option for postexposure prophylaxis of MERS requires study.

TREATMENT

Supportive Care

No specific antivirals are proven effective for any coronavirus infection, and good supportive treatment is the mainstay for clinical management. Syndrome-related symptomatic therapy is indicated. In infections leading to severe respiratory illness leading to ARDS, as occurs with SARS and MERS, ventilation using a lung-protective strategy with small tidal volume is advised (238). The use of corticosteroids in viral pneumonia leading to ARDS is in general to be avoided, except in patients with refractory shock or other clinical indication for corticosteroid use. In a retrospective cohort study of patients with SARS, corticosteroid use was associated with worsened outcome and a prolongation of viral shedding (239).

Antiviral Treatment

Because of the clinical severity and lack of prior data for SARS, various treatments have been attempted for SARS patients. During the outbreak of 2002–2003, many patients were treated with intravenous or oral ribavirin, and those with severe disease also received systemic corticosteroids, often in high doses. A systematic review of different treatments used for SARS found evidence for inhibition of SARS-CoV *in vitro* by ribavirin, lopinavir, and type I IFN (239). Observational trials of convalescent plasma or intravenous immunoglobulin (IVIG), type I IFN, ribavirin, and lopinavir/ritonavir were inconclusive. Corticosteroid therapy worsened clinical outcomes (239) and increased duration of virus shedding. Pegylated IFN had therapeutic efficacy in macaques, and IFN-alfacon1 may have had some beneficial effect in a preliminary clinical study in humans (240).

Potentially useful drugs for treatment of MERS have been identified on the basis of the *in vitro* experimental animal studies and, where available, any human clinical studies (240–242). MERS-CoV is inhibited by type I IFN *in vitro*, and IFN-alpha2b combined with high doses of ribavirin modestly reduced viral titers and lung pathology in experimentally infected rhesus macaques (241). Lopinavir/ritonavir or IFN-beta1b treatment improved outcome in common marmosets infected with MERS-CoV, but there are criticisms about the experimental model used (242). One observational study of IFN-alpha2b combined with ribavirin treatment in several severely ill patients with MERS found evidence of lower mortality at day 14 but not at day 28, and other observational studies have not yielded encouraging results (210). A number of other compounds (e.g., chloroquine, chlorpromazine, loperamide, lopinavir) have shown inhibitory effects of MERS-CoV *in vitro*, but their effectiveness *in vivo* remains unclear (reviewed by 143).

Passive immunotherapy with neutralizing antibodies from convalescent plasma, equine or camel immune sera, anti-S monoclonal antibodies, and polyclonal human antibodies from transgenic cows have also been identified as potentially beneficial (244). Passive immunotherapy has shown some promise in animal models of SARS (245), and a meta-analysis of clinical trials demonstrated a possible reduction of mortality (244). Clinical trials in MERS patients are anticipated with one or more of these immunotherapeutics.

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Arthropod-Borne Flaviviruses

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In 1901 the prototype flavivirus disease, yellow fever, was the first human illness shown to be caused by a filterable virus, and, in 1927, it became the first member of the flavivirus family to be isolated. The *Flaviviridae* derive their name from yellow (*flavus*, Latin) fever. From the medical perspective, the flaviviruses are the most important group of arthropod-borne viruses (arboviruses). Dengue fever and dengue hemorrhagic fever (DHF) are major causes of human morbidity worldwide. Yellow fever remains an epidemic threat in Africa and South America. Since its introduction into North America, West Nile virus (WNV) has caused annual outbreaks of encephalitis and febrile illness in North America and has spread throughout the Americas as far south as Argentina; Japanese encephalitis (JE) remains a major cause of viral encephalitis in Asia.

VIROLOGY

Classification

The *Flaviviridae* constitute a structurally unique virus family of positive-sense, single-stranded RNA viruses, divided into the genera *Flavivirus*, *Pegivirus*, *Pestivirus*, and *Hepacivirus*. There are approximately 60 distinct agents in the *Flavivirus* genus, most of which are transmitted by arthropods (mosquitoes or ticks), and more than half are associated with human disease. Classification of viruses within the *Flavivirus* genus has been traditionally based on antigenic distinctions, and each of the 60 virus species represents a distinct "species" or serotype. Using the neutralization test, flaviviruses have been classified into at least eight antigenic complexes, of which six contain human pathogens (1). More recently, nucleotide sequencing has assumed a prominent role in determining relationships among viruses and identifying which are termed "species." The current virus species and their groupings are shown in Table 1. Twelve virus groups have been identified, and an additional group of viruses has been found in insects (insect-specific flaviviruses). Recently a large number of flaviviruses have been identified from either mosquitoes or ticks that do not replicate in vertebrate hosts and have no apparent pathogenic effects in humans (2). While it is clear that these are related to currently identified flaviviruses, it is unclear if they are members of the *Flavivirus* genus. For this reason, they are not considered further in this chapter. The classification of the tick-borne flaviviruses is still being debated (3).

The viruses within the tick-borne, dengue, and Japanese encephalitis serocomplexes are most closely related to each other, sharing up to 77% of their amino acid sequences, whereas homology across the serocomplexes is only 40% to 45%. Immunological cross-protection between viruses is not observed for agents with $<70\%$ sequence homology, but incomplete or partial cross-protection may be present across closely related viruses within a serocomplex. Many closely related members of a serocomplex are allopatric species (i.e., subdivided into geographically isolated populations), so that immunological interactions do not occur naturally. Exceptions with overlapping geographic distributions (e.g., dengue serotypes, Japanese encephalitis West Nile) will be noted in the text.

Flaviviruses undergo high rates of mutation characteristic of RNA viruses, and each species consists of multiple genetic variants. Genetic variation is tempered by the requirement to maintain sufficient genetic stability to ensure fitness in hosts and vectors belonging to different phyla. Recombination within species contributes to strain variation of hyperendemic viruses (such as dengue) that have high rates of coinfection of hosts and vectors (4). An analysis of flavivirus phylogenetic changes by comparing complete E glycoprotein nucleotide sequences showed that the mosquito-borne flaviviruses (like dengue) have undergone explosive genetic diversification within the last 200 years, whereas the more primitive tick-borne viruses evolved slowly (5), probably due, in part, to the different ecologies of the viruses. The recent evolution of dengue viruses is associated with the expanding size and dispersal of human host and vector populations, while the tick-borne agents are transmitted in stable environments between small terrestrial mammal hosts and vectors with a long reproductive cycle.

Composition

Flavivirus particles are approximately 50 nm in diameter and have a spherical nucleocapsid surrounded by a lipid-bilayer envelope with small surface projections, comprised of E glycoprotein dimers anchored to the virus membrane. The envelope protects the genome from cellular nucleases. Lipases, organic solvents, and detergents disrupt the virus envelope and inactivate flaviviruses.

The flavivirus genome is a single strand of RNA of positive polarity containing approximately 11,000 nucleotides (6) and is composed of a short 5' noncoding region, a single

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TABLE 1 List of members and tentative members of genus *Flavivirus*

Vector	Group	Species	Subtype	Comments	Genomic sequence
Tick-borne	Mammalian tick-borne	*Gadgets Gully*			+
		Kadam			+
		Karshi			+
		Langat			+
		Kyasanur Forest disease			+
			Alkhumra	Genetic variant of Kyasanur Forest disease virus	+
		Langat			+
		Louping ill	British		+
			Irish		+
			Greek		+
			Spanish		+
			Turkish		+
		Omsk haemorrhagic fever			+
		Powassan			+
			Deer tick	Genetic variant of Powassan virus	+
		Royal Farm			+
		Tick-borne encephalitis	Central European		+
			Far eastern		+
			Siberian		+
Tick-borne	Seabird tick-borne	*Meaban*			+
		Saumarez Reef			+
		Tyuleniy			+
Mosquito-borne	Aroa	*Aroa*			+
		Bussuquara			+
		Iguape			+
		Naranjal			+
Mosquito-borne	Dengue	*Dengue type 1*			+
		Dengue type 2			+
		Dengue type 3			+
		Dengue type 4			+
Mosquito-borne		*Kedougou*			+
Mosquito-borne	Japanese encephalitis	Alfuy			+
		Cacipacore			+
		Koutango			
		Japanese encephalitis			+
		Murray Valley encephalitis			+
		St. Louis encephalitis			+
		Usutu			+
		West Nile			+
			Kunjin		+
		Yaounde			
Mosquito-borne	Kokobera	*Kokobera*			+
		New Mapoon		Tentative species	
		Stratford			+

(*Continued*)

TABLE 1 List of members and tentative members of genus *Flavivirus* (*Continued*)

Vector	Group	Species	Subtype	Comments	Genomic sequence
Mosquito-borne	Ntaya	*Bagaza*			+
		Ilheus			+
		Israel turkey meningoencephalomyelitis			+
		Ntaya			+
		Rocio			+
		Tembusu			+
Mosquito-borne	Spondweni	*Spondweni*			+
		Zika			+
Mosquito-borne	Yellow fever	*Banzi*			
		Bouboui			
		Edge Hill			
		Jugra			
		Saboya			
			Potiskum		
		Sepik			+
		Uganda S			
		Wesselsbron			+
		Yellow fever			+
No known vector	Entebbe bat	*Entebbe bat*		Genetically Entebbe bat group is in the yellow fever group	+
			Sokoluk		+
		Yokose			+
No known vector	Modoc	*Apoi*			+
		Cowborne Ridge			
		Jutiapa			+
		Modoc			+
		Sal Vieja			
		San Perlita			
No known vector	Rio Bravo	*Bukalasa bat*			
		Carey Island			
		Dakar Bat			
			Batu Cave		+
		Montana myotis leukoencephalitis			+
		Phnom Penh			+
		Rio Bravo			+

long open reading frame containing more than 10,000 nucleotides, and a 3' noncoding region (3'NCR) of variable length that is usually devoid of poly A tracts. The long open reading frame encodes 3 structural proteins at the 5' end, which are the capsid (C), premembrane (prM), and envelope (E) proteins. These are followed downstream by 7 nonstructural (NS) proteins in the sequence NS1, NS2A-NS2B-NS3-NS4A-NS4B-NS5 (Table 2). The structural proteins are incorporated into the mature virion, whereas the NS proteins play various roles in virus replication and polypeptide processing. The polyprotein is co- and post-translationally cleaved to yield the individual proteins. Translation occurs at the rough endoplasmic reticulum (ER), so that the prM, E, and NS1 proteins are translocated, whereas the others remain on the cytoplasmic side of the host-cell membrane. Cell-associated virions within the ER are morphologically identical to extracellular particles.

The capsid protein interacts with RNA to form the virion nucleocapsid. The prM glycoprotein forms an intracellular heterodimer, stabilizing the E polypeptide during exocytosis.

TABLE 2 Flavivirus proteins and their known or suspected functions

Protein	Location	Mol mass (kDa)	Function	
			Replication	Immunity
C	Capsid	9–12	Associates with RNA in nucleocapsid	T cell epitopes
prM	Premembrane (immature virion)	18–19	Chaperone for E protein; proper folding and stabilization during virus maturation and exocytosis	Protective immunity (minor role, induces low titer neutralizing antibody)
M	Membrane (mature virion)	7–9	Control of fusion events (with E)	T-helper epitope?, induces low titer neutralizing antibody
E	Envelope	55–60	Receptor binding; mediation of membrane fusion in endosome and virus internalization; dissociation of nucleocapsids	Neutralizing (protective) antibody, CTL epitope(s), Th epitopes, immune enhancement, hemagglutination inhibition antibody
NS1	Nonstructural	42–50	Involved in RNA replication and virion assembly/maturation	Antibody, complement-dependent cell lysis; CTL epitopes
NS2A		18–22	Assembly; release	? CTL epitope; interferon antagonist for West Nile virus
NS2B		13–15	Central domain associated with functional NS3 protease	
NS3		67–70	Serine protease (posttranslational processing of viral proteins), helicase, nucleoside triphosphatase, RNA 5'-triphosphatase; RNA capping	Nonneutralizing antibody; CTL epitopes
NS4A		16	Integral membrane protein; induces endoplasmic reticulum membrane rearrangement; regulates ATPase activity of NS3 helicase; part of replication complex	? CTL epitope; induces autophagy
NS4B		27–28	Integral membrane protein; part of replication complex	? CTL epitope; RNA interference; formation of stress granules; unfolded protein response; interferon antagonist for West Nile and dengue-2 viruses, and others?
NS5		104–106	RNA-dependent RNA polymerase, methyltransferase, RNA capping	Nonneutralizing antibody; CTL epitope; interferon antagonist for Langat and Japanese encephalitis viruses; induction of IL-8

The prM protein is cleaved before virus release from the cell, leaving a small M structural protein anchored in the virus envelope, and releasing the larger 18–19 kDa "pr" glycopeptide segment into the extracellular medium.

The E glycoprotein contains antigenic determinants for hemagglutination and neutralization. Antibodies directed at E and prM determinants also mediate the phenomenon of antibody-dependent enhancement (ADE) of infection in the pathogenesis of dengue hemorrhagic fever (7–9). The E protein is also involved in attachment to cells and fusion of the viral and host-cell membrane during virus entry into the cell, and thus is a major factor in virus virulence. Mutations in the E gene are often responsible for significant alterations in biologic functions of the virus and may affect virulence; however, virulence is multigenic, and evidence suggests that most virus-encoded proteins and 3'NCR contribute to pathogenicity.

The three-dimensional crystallographic structure of the E glycoprotein reveals a head-to-tail dimer composed of a 170-Å-long rod anchored to the membrane at its basal end with its long axis parallel to the virion surface (10) and is typical of a class II fusion protein characteristic of alphaviruses and flaviviruses. The C-terminus resembles an immunoglobulin constant domain and is connected by a hinge region to a central portion of the molecule (domain I) with up-and-down topology consisting of eight antiparallel strands and containing the N-terminus. Two long loops (domain II) extend outward from this part of the protein and play a role in dimerization of the molecule. A conserved stretch of 14 amino acids at the end of one of the domain II loops is responsible for fusion of the viral envelope and the membrane of the host cell (11). The fusion event is acid-dependent and occurs within endosomal vesicles, releasing the uncoated nucleocapsids into the cytoplasm of the infected cell. The fusion process also results in an irreversible rearrangement of the E protein into a trimeric form. Domain III contains a ligand(s) involved in binding-cell receptors. An Arg-Gly-Asp (RGD) protein sequence in domain III is involved in attachment to glycosaminoglycan receptors (e.g., heparan sulfate) on cell membranes, but other ligand-

receptor interactions probably also occur. To date, no cell receptor has been conclusively proven for any flavivirus. Discontinuous (conformational) neutralization determinants are scattered across all three structural domains but tend to be located on the accessible surface of the E protein, with critical determinants located on domain III and near the fusion peptide region. In addition to infectious virions, several noninfectious subvirus structures are present on, or are released from, infected cells.

The NS1 protein both is released extracellularly and is associated with the plasma membrane of infected cells in the form of a dimeric structure anchored to glycosylphosphotidylinositol (12). The secreted form is antigenic ("soluble complement fixing" antigen), containing both virus-specific and cross-reactive epitopes, and for dengue is also detectable in blood for diagnostic purposes. Antibodies to NS1 do not react with the virion and exhibit no neutralizing activity. Protective immunity is mediated by antibody- and complement-mediated lysis of cells bearing NS1 targets (13).

NS3 functions as a serine protease, involved in posttranslational cleavage of the virus polyprotein, and also has RNA helicase, NTP phosphatase, and RNA triphosphatase activities. The protein is present in cell membranes, stimulates virus-specific T-cell responses, and is a target for attack by cytotoxic T cells containing multiple dominant epitopes for CD4+ and CD8+ T lymphocytes in both mice and humans (14). Like NS3, the NS5 protein is also highly conserved; it functions both as the RNA-dependent RNA polymerase in virus replication and as a methyltransferase in 5' cap methylation. The functions of the other NS proteins in replication are poorly defined. NS2A, NS4A, and NS4B function as interferon antagonists for West Nile and dengue-2 viruses (15), while NS5 has the same activity for Langat and JE viruses.

Replication Strategy

Flaviviruses enter cells by attachment to heparan sulfate or other, as yet undefined, receptors, followed by uptake of the virus into cells by receptor-mediated endocytosis in clathrin-coated vesicles. Subsequent low-pH-induced fusion of the viral envelope and endosomal membrane involves an acid-mediated change in the configuration of Domain II of the E glycoprotein. The nucleocapsids are released into the cytoplasm, after which the uncoated genomic mRNA is translated into a polyprotein that includes the polymerase, protease, and helicase enzymes (NS5 and NS3) required for continued replication. This genomic RNA also forms the template for synthesis of complementary negative RNA strands, which serve in turn as templates for full-length plus strands. The progeny genomic mRNA serves two functions. First, it synthesizes additional negative strands that serve as templates for more progeny-genomic RNA that will be assembled into virions, and, second, it translates the mRNA to yield additional nonstructural and structural viral proteins required for continued replication and virion assembly. Assembly of virus particles occurs in close association with ER. Virus particles are transported through the ER to the plasma membrane, where they are exocytosed.

Propagation in Cell Culture

Many cell-culture types of human, monkey, rodent, swine, and avian tissue origins are useful for the replication and assay of flaviviruses (Table 3). Monkey kidney cells (Vero, LLC-MK2), hamster kidney (BHK-21), porcine kidney (PS), human adrenal carcinoma (SW-13), as well as primary chick or duck embryo cells, have been widely used. Virus titers of 10^6 to 10^8 tissue-culture-infective doses ($TCID_{50}$), or PFU per milliliter, are readily achievable for most viruses in the genus. Both cytopathic effects (CPE) and plaque/focus formation are observed in these cells, but these vary considerably with the specific virus and host cell (see below). Flavivirus replication in cultured cells may also be measured by detection of antigen or nucleic acid in the cytoplasm of cells by immunocytochemistry or in supernatant fluids by enzyme-linked immunosorbent assay (ELISA), complement fixation (CF), or reverse-transcriptase polymerase chain reaction (RT-PCR).

Mosquito cell cultures, including C6/36 *Ae. albopictus* cells, are widely used for virus isolation or assay. Cytopathic effects (CPE; syncytium formation) and plaque formation occur after infection with some viruses, but others are noncytopathic. Tick-borne flaviviruses replicate in tick cell cultures without causing CPE. The mosquito-borne group viruses replicate well in mosquito, but not tick cell, cultures; the converse is true for the tick-borne group viruses (16), whereas the no-vector group does not replicate in either. While most flaviviruses replicate in vertebrate cell cultures, some flaviviruses replicate in mosquito or tick cells only (2). Some viruses can replicate to some extent both in tick and mosquito cell cultures (e.g., WNV and St. Louis encephalitis [SLE] virus), which correlates with occasional field observations.

Multiplication in cell culture includes a rapid absorption phase, followed by an eclipse phase of approximately 10 to 12 hours, after which infectious virus first appears and enters a log phase of replication lasting 18 to 24 hours. Peak titers in fluid phase cultures may appear after 24 hours at high multiplicities of infection, but multiplication of some flaviviruses is considerably slower. Persistent infection without cytopathology has been demonstrated in a variety of arthropod and vertebrate cell lines. Progeny virus from such cultures may have altered antigenic properties, reduced virulence, or temperature sensitivity.

Inactivation by Physical and Chemical Agents

Flaviviruses are rapidly inactivated by ionic and nonionic detergents, trypsin, ultraviolet light, γ-irradiation, formaldehyde, β-propiolactone, ethyleneimine, and most disinfectants, including chlorine, iodine, phenol, and alcohol. The viruses are optimally stable at temperatures below –70°C, and they are rapidly inactivated in blood or other liquids within 30 min at 56°C. Flaviviruses are optimally stable at pH 8.4 to 8.8 and, with the exception of some tick-borne viruses, are rapidly degraded at low pH. Sensitivity to acid, bile, lipases, and proteases in the gastrointestinal tract generally precludes infection by the oral route, although tick-borne encephalitis may be acquired by ingestion of milk.

BIOLOGY

Host Range and Route of Infection

The host range of specific flaviviruses varies considerably by individual agent (reviewed by Weaver and Barrett) (17). In general, laboratory rodents (mice and hamsters) are susceptible to infection and develop lethal encephalitis after intracerebral inoculation (Table 3). Many hosts that get clinical disease are dead-end hosts due to low viremia such that biting mosquitoes/ticks cannot be infected during feeding (Table 3). Flaviviruses all have transmission cycles involving wild vertebrate species and infect their natural vertebrate hosts via the bite of blood-feeding arthropods.

TABLE 3 Host range and cell culture systems most useful for isolation and assay of flaviviruses, and vectors demonstrating infection and transmission in the laboratory

Virus[a]	Laboratory host (lethal infection)[a]	Economically important animal[a,f]	Nonhuman primate[a]	Cell culture	Arthropod vector
CEE virus[e]	SM (i.c., i.p.), WM (i.c.), WH (i.c.), CE	Goat, sheep (encephalitis, i.c.)	Rhesus, cynomolgus (encephalitis, i.c.)	Vero, CE, BHK, HeLa, PS	*Ixodes* ticks
DEN virus	SM (i.c.)[b], WM (i.p.)[d]		Rhesus (s.c., viremia)	LLCMK2, BHK, PMK, C6/36	*Aedes* and *Toxorhynchites* mosquitoes
JE virus[e]	SM (i.c., i.p.), WM (i.c.), CE	Horse (encephalitis, i.c., i.n.), pig (viremia, s.c.)		Vero, LLCMK2, CE, C6/36	*Culex* and *Aedes* mosquitoes
KFD virus[e]	SM (i.c., i.p.), SH (i.c.), WM (i.c., i.p.)		Rhesus (viremia, i.c., s.c.)	BHK, PMK, LLCMK2, Vero, HeLa, CE	*Haemaphysalis* ticks
OHF virus[e]	SM (i.c., i.p.), WM (i.c., i.p.), WH (i.c.), GP (i.c.)	Muskrat (hemorrhagic disease)	Rhesus (encephalitis, i.c.)	BHK, PS, HeLa, CE	*Dermacentor* ticks, *Culex* and *Aedes* mosquitoes
MVE virus[e]	CE, SM (i.c., i.p.), WM (i.c., i.p.), WH (i.c., i.p.)	Sheep (encephalitis, i.c.)		CE, PS, BHK, Vero, LLCMK2	
POW virus[e]	SM (i.c., i.p.), WM (i.c., i.p.)		Rhesus (encephalitis, i.c.)	BHK, LLCMK2, Vero, PS	*Ixodes* ticks
ROC virus[e]	SM (i.c., i.p.), WM (i.c., i.p.), SH (i.c., i.p.), WH (i.c., i.p.)			DE, Vero, PS, BHK, MA-104	*Aedes* mosquitoes
SLE virus[e]	SM (i.c., i.p.), SM (i.c., i.p.); WH (i.c.), WH (i.c.)		Rhesus (encephalitis, i.c.)	DE, CE, Vero, LLCMK2, PS, BHK, SW13	*Culex* mosquitoes
WN virus[e]	SM (i.c., i.p.), WM (i.c., i.p.), chick (i.c., s.c.), WH (i.c.)	Horse (viremia)	Rhesus (encephalitis, i.c.)	CE, BHK, Vero, LLCMK2, PS, C6/36	*Culex*, *Aedes*, and *Anopheles* mosquitoes
YF virus	SM (i.c., i.p.), WM (i.c.), WH (i.c.), WH (i.p.)[c]		Rhesus, cynomolgus (encephalitis, i.c.; hepatitis, s.c.)	AP 61, Vero, LLCMK2, BHK, SW13, C6/36	*Aedes* and *Toxorhynchites* mosquitoes

[a]Abbreviations: CEE, Central European encephalitis; DEN, dengue; JE, Japanese encephalitis; KFD, Kyasanur Forest disease; OHF, Omsk hemorrhagic fever; MVE, Murray Valley encephalitis; RSSE, Russian spring-summer encephalitis; SLE, St. Louis encephalitis; WN, West Nile; YF, Yellow fever; SM, suckling mouse; SH, suckling hamster; WM, weaned mouse; WH, weaned hamster; GP, guinea pig; i.c., intracerebral route; i.p., intraperitoneal route; i.n., intranasal route; s.c., subcutaneous route; DE, primary duck embryo; CE, primary chicken embryo; BHK, baby hamster kidney; SW13, human adrenal carcinoma; Vero, LLCMK2, MA-104, monkey kidney; PS, porcine kidney; C6/36, *A. albopictus*; AP61, *A. pseudoscutellaris*.

[b]Often requires adaptation by brain serial passage.

[c]Often requires adaptation by liver serial passage.

[d]AG129 and nude mice.

[e]Humans are dead-end hosts due to insufficient viremia for a mosquito/tick to pick up the virus during feeding.

[f]All economically important animals are dead-end hosts.

Several mosquito-borne zoonotic viruses in the JE complex cause central nervous system (CNS) disease in both humans and domesticated livestock, particularly horses. Viruses in this group also infect wild birds, which serve as intermediary hosts in transmission cycles. In a balanced virus ecosystem, wild birds infected with flaviviruses typically do not suffer overt clinical signs of disease (e.g., Japanese encephalitis virus) but sometimes this is not the case (e.g., West Nile virus in American crows). Rodents, insectivores, and birds are involved in transmission of TBE complex viruses, typically without causing disease in these hosts. Tick-borne flaviviruses also cause zoonotic disease in humans and domestic livestock. Kyasanur Forest disease virus (a member of the TBE complex) causes fatal illness in nonhuman primates, and Omsk hemorrhagic fever causes illness and death in an exotic rodent species (muskrats) introduced from North America to Russia. Yellow fever and dengue viruses are principally human pathogens but also infect certain nonhuman primates and, in the case of yellow fever, produce a similar illness (hepatitis) in certain neotropical monkey species.

Arthropod-borne flaviviruses are infectious for mosquito and tick vectors by the oral route and replicate to high titers in arthropod tissues. Of the approximately 60 flaviviruses, 16 have no known arthropod vector and are presumably transmitted directly between specific vertebrate-reservoir host-species, including rodents and bats. Contact infection of these hosts may be transmitted by the respiratory or oral route or by bites. Only isolated cases of human illness caused by a rodent-borne (Modoc virus) or bat-borne virus (Dakar bat virus) have been reported, but this observation may reflect lack of exposure rather than host-range restriction.

Arthropod Infection

Medically important flaviviruses are transmitted by the bite of infected mosquitoes or ticks, which are true biologic (as opposed to mechanical) vectors. After ingestion of a blood meal containing virus, replication occurs in midgut epithelial cells, and virus is released into the hemolymph, whence it invades the salivary gland. Ultimately virus is secreted in saliva during refeeding on a susceptible vertebrate host. The interval between the ingestion of virus-containing blood and the salivary secretion of virus (extrinsic incubation period) is critical to transmission, because this interval must not exceed the lifespan of the arthropod. Increased temperature shortens the extrinsic incubation period and may thus increase the rate of virus transmission in nature. In general, flaviviruses do not induce pathologic changes in the arthropod vector.

Mosquito-borne flaviviruses are amplified in nature by sequential passage through vectors and vertebrate hosts. Because of the small volume of blood ingested by the vector, the threshold concentration of virus in vertebrate blood required to infect 1% of vectors is quite high ($\backsim$3 to 5$\log_{10}$ infectious units/mL). Hosts that do not circulate virus at high titer, the so-called "dead-end hosts," are excluded from transmission cycles. This is true for human beings in the case of most flaviviruses, notable exceptions being dengue and yellow fever. Ticks imbibe much larger volumes of blood over a long period of time. This adaptation requires down-regulation of the host's coagulation, inflammatory, and immunological responses by proteins contained in tick saliva (18). TBEVs are transmitted efficiently from infected to uninfected ticks via tick saliva shared during simultaneous feeding on a vertebrate host. WNV can be transmitted from infected to uninfected mosquitoes feeding on birds.

Vertical transmission in arthropods is an important mechanism for overwinter survival of some flaviviruses. Flaviviruses infect the genital tract of male and female mosquitoes, and virus enters the ovum at the time of fertilization. Venereal transmission of flaviviruses from male to female mosquitoes also occurs. The mechanism of transovarial transmission of tick-borne flaviviruses remains to be elucidated.

PATHOGENESIS

Virus-Host Interactions

Human disease caused by flaviviruses may be classified syndromically as either (i) CNS infection; (ii) hemorrhagic fever; or (iii) fever-arthralgia with or without rash. By contrast, mice develop encephalitis after infection with most flaviviruses, with the exception of mice deficient in both alpha/beta and gamma interferon receptors where some strains of yellow fever and dengue viruses induce viscerotropic disease in this model (19–22), and have been widely used as a model for study of pathogenesis. After the bite of a mosquito or tick, flaviviruses replicate in dendritic cells with subsequent spread to regional lymph nodes, whence they are transported via lymphatics to the thoracic duct and then to the bloodstream. Langerhans' dendritic cells are important for transport of virus to lymphoid tissues (23, 24). After inoculation of virus in mosquito saliva, replication in local tissues may occur for several hours before vascular dissemination. Components of mosquito or tick saliva appear to facilitate flavivirus transmission by interfering with aspects of both innate and adaptive immunity (25, 26). After dissemination to, and replication in, extraneural tissues, large amounts of virus are shed directly into the blood. Viremia is terminated around 1 week after infection by both innate and adaptive immune responses (27). Depending on the specific agent, extraneural cells and tissues involved in flavivirus replication include macrophages and other lymphoid cells, skeletal muscle and myocardium, smooth muscle, and endocrine and exocrine glands. Major differences have been noted between specific cell types in their ability to sustain infections. Although no comprehensive analysis of flavivirus-cell interactions *in vivo* is possible, the special interaction of dengue viruses with dendritic cells and monocytes/macrophages, of dengue and yellow fever viruses with hepatocytes, and of the encephalitis viruses with neural cells underlie pathogenesis.

Invasion of the CNS appears to be closely linked to virus replication in extraneural sites and to the level of viremia. Factors that impair the blood-brain barrier, including immaturity, traumatic disruption of the barrier, or concurrent infection of the brain with unrelated agents, potentiate neuroinvasion, and encephalitis (28). The mechanism by which flavivirus particles enter the central nervous system during natural infection remains uncertain. Postulated mechanisms include entry via leukocytes, direct entry across the blood-brain barrier, and entry by retrograde axonal transport via the peripheral nervous system (27). In addition, there is evidence that the innate immune response can either contribute to inhibition or promotion of neuroinvasion (29–31). In the mouse, WNV RNA can be detected in the brainstem and spinal cord within the first 2 days of infection, and viral antigen was observed in the cortex, hippocampus, and choroid plexus by the third day, suggesting multiple routes of CNS invasion (32). Although neuroinvasive flaviviruses can be identified from most regions of the central nervous system, a predilection for involvement of the

anterior horn cells of the spinal cord, as well as the thalamus, substantia nigra, pons, and cerebellum, accounts for the acute flaccid paralysis and movement disorders clinically observed following infection in monkeys and humans.

Persistence of immunoglobulin M antibody following acute infection has been noted for some flaviviruses, raising the question of persistent infection (33). Several animal models have demonstrated viral persistence, and chronic infections sometimes associated with progressive neuropathology have been observed (33). In humans, persistent infection with recurrent neurologic disease has been reported in patients following JE and TBE.

Age-related resistance in mice is manifested around 1 to 2 weeks of age with maturity of the blood-brain barrier, at which time parenteral injection of virus elicits inapparent infection, whereas intracerebral inoculation still causes lethal encephalitis. In humans, some flaviviruses (e.g., SLE virus, WNV) cause severe disease principally in the elderly, whereas others have a bimodal distribution with an excess of cases at both age extremes (e.g., JE and Murray Valley encephalitis [MVE]).

The genetic background of the host plays an important role in susceptibility to infection (34). In the mouse, presence of a truncated form of the 2'-5'-oligoadenylate synthetase (OAS) gene located on chromosome 5 increases susceptibility to flavivirus encephalitis (35). Although a similar mutation has not been observed in humans, an increased frequency of an OAS-like allele that contained a splice-enhancer site was observed in patients hospitalized with WNV infection (36). Humans with a deletion in the CCR5 gene may be at higher risk for a poor outcome following WNV infection (37, 38).

Immune Responses

Innate immune responses, including natural killer (NK) cells, alpha/beta interferon (IFN-α/β), and other cytokines, and nitric oxide production by macrophages, are the first line of defense and also modulate the acquired, virus-specific immune responses that rapidly follow infection (39). In recent years a considerable body of information is being obtained about the innate immune response, particularly to WNV and dengue (40, 41), as a paradigm for the flavivirus genus as a whole. These studies show that once a flavivirus has infected a cell, there is a cascade of intracellular signaling that results in production of IFN-α/β. This, in turn, induces intracellular antiviral responses that subsequently initiate the adaptive immune response. Specifically, once the virus uncoats inside the cell, pattern-recognition receptors, such as toll-like receptors (TLRs), recognize double-stranded and single-stranded RNA (e.g., TLR-3, TLR-7, and TLR-8) and result in induction of IFN-α/β. Similarly, retinoic-acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) recognize viral dsRNA and lead to induction of IFN-β. IFN-α/β is secreted by virus-infected cells and triggers an "antiviral state" in surrounding cells via signaling pathways involving Janus Kinase and signal transducer and activation of transcription (JAK/STAT) molecules that lead to expression of a variety of IFN-stimulating genes that have antiviral activity. The situation is complicated because it has been found that various cell types in the body vary in their ability to mount an antiviral response. Not surprisingly, flaviviruses have developed multiple approaches to overcome the intracellular antiviral responses and signaling cascades by utilizing nonstructural proteins in various roles (Table 2), and this "war" between the host's innate immune response and the functions of viral proteins will ultimately determine whether or not a virus infection results in clinical disease. Abortive (subclinical) infections, which are the norm, are the presumed result of robust innate- and acquired-immune responses that out-pace virus-mediated cell damage, as well as virus-induced apoptotic cell death that limits the progression of infection in the host.

Antibodies appear to play the primary role in protection against reinfection, while both antibodies and cellular immune responses are responsible for clearance of virus-infected cells and recovery from active infection. Neutralizing antibodies directed against determinants on the E glycoprotein provide long-lived, virus-specific protection against reinfection. Thus, infection by one virus gives lifelong immunity to that particular virus but not to other flaviviruses. Infection also elicits antibody responses against the NS1 nonstructural protein that decorates the surface of infected cells, and anti-NS1 antibodies may protect against reinfection and play a role in recovery from infection.

Pathologic events *in vivo* are principally the result of direct virus injury, although immunopathologic mechanisms are implicated in some preclinical models. The extent to which the host's immunologic response to infection plays a role in pathogenesis in human infection is uncertain, except in the case of DHF (see below). In flavivirus encephalitis, neuroinvasion and rapid accumulation of viral antigen in the critical target tissues occur late in the course of infection and may potentially elicit inflammatory responses that enhance lesions and accelerate death of infected cells. NK cells and CTLs lyse infected neurons, and CTLs interact with infected astrocytes (42). Infection of these cells in the brain appears to result in an enhanced expression of MHC-I, with increased CTL recognition of infected cells. The increase in MHC-I molecules on the infected cell surface may be induced by interferon or may be a result of flavivirus peptide transport to the ER. In addition to cell-mediated clearance, antibodies can be shown to cause early death in mice infected with yellow fever and MVE viruses (43, 44). This effect is attributed to complement-mediated cytolysis of infected cells. Infiltration of neutrophils into perivascular areas of the infected brain, in response to tumor necrosis factor alpha (TNF-α) and neutrophil chemotactic factor, results in the expression of inducible nitric oxide synthase activity, which appears to increase pathogenesis (45), possibly by inducing apoptosis. An additional possible immunopathological mechanism is antibody binding to glucosyl-phosphatidylinositol-anchored NS1, followed by signal transduction, activation of superoxide anions, and apoptosis (12). The relevance of such experimental observations to human disease is an area of active research, including the effects of active and passive immunization against heterologous flaviviruses on the course and outcome of neurologic infection. There is evidence for cross-protection between flaviviruses in humans, whereas cross-reactive immunopathological responses have not been clearly defined except in the case of dengue disease.

The most important example of immunopathologic response in humans is antibody-dependent enhancement (ADE) of flavivirus replication in Fcγ-receptor-bearing peripheral blood monocytes (7). This phenomenon is thought to determine the severity of dengue (see section on DHF below), although excessive pro-inflammatory innate immune responses may also contribute to severe dengue disease (46). Antibody attack against molecular mimics has also been postulated to play a role in dengue pathogenesis. The dengue E protein contains a 20-amino-acid region of homology with plasminogen, the mediator of fibrinolysis, and

anti-E antibodies might interfere with fibrinolysis and contribute to disseminated intravascular coagulopathy (47).

Flavivirus NS proteins are processed in a major histocompatibility class I (MHC-I)-dependent fashion resulting in serotype-specific and cross-reactive T-cell clones. Cytotoxic T cells, recognizing NS proteins in infected cells, are involved in postinfection virus clearance. The structural proteins, particularly E and prM, as well as NS1, stimulate MHC-II-restricted responses and generate antibodies protective against subsequent infection. However, the E and NS1 proteins also play a role in the generation of cytotoxic T lymphocytes (CTLs).

Virus-Specific Factors in Virulence

Differences have often been noted in the expression of disease caused by the same flavivirus among individual patients, among geographic regions, or between the early and late phases of an epidemic. These variations may be due in part to strain-specific differences in virulence genes. Virulence is multigenic, including mutations in the E gene affecting attachment and uncoating, and/or in nonstructural genes and 3'NCR affecting replication rate. Neurovirulence determinants in the E protein genes of yellow fever virus, JE virus, and tick-borne encephalitis virus (TBEV) have been partially mapped by comparing virulent strains and attenuated vaccines derived from wild type virus, or by attenuated neutralization escape mutants and mutants unable to bind brain-membrane receptors (48, 49). E-gene virulence determinants include (i) amino acid residues at the interface of domains I and II involved in reconfiguration of the E protein and trimer formation under acidic conditions in the endosome; (ii) residues (E98 to E120) in the fusion domain at the end of domain II; (iii) residues (E305 to E315) in the upper lateral surface of domain III involved in virus-cell attachment (49); and (iv) residues in the C-terminal stem-anchor region involved in E-protein conformational changes during fusion. Other studies have identified mutations in nonstructural genes, NS1, NS2A, NS3, NS4A, NS4B, and NS5 and in the 5' and 3'UTR that result in a decrease in virulence by downregulating the rate of virus replication.

FEATURES OF SPECIFIC FLAVIVIRUSES

Viruses Causing Central Nervous System Infection

Japanese Encephalitis Virus

Biology

JE virus is the prototype of an antigenic complex that includes SLE and MVE viruses, WNV, and several other flaviviruses of lesser medical importance (Table 1). Although JE virus represents a single antigenic serotype (50), fine distinctions of no recognized clinical importance exist between JE virus strains serologically, genetically, and by biological assays, such as cross-protection in mice and neurovirulence. Five distinct genotypes (genetic clusters) of JE virus exist: genotype I includes isolates from Cambodia and northern Thailand; genotype II includes isolates from Indonesia, southern Thailand, and Malaysia; genotype III includes isolates from temperate regions of Asia: Japan, China, Taiwan, India, Nepal, Sri Lanka, and the Philippines.

Transmission of JEV is epidemic in temperate zones with the majority of cases occurring in summer months, whereas transmission is endemic in tropical zones and occurs year-round at lower rates. Genotypes III and GI-b are found in temperate areas, while GI-a and GII are found in tropical areas (51). Genotype IV includes certain isolates from Indonesia only. Genotype V has been isolated only on rare occasions and has recently re-emerged after almost 60 years of undetected virus circulation in Korea, China, and Tibet (52, 53, 54). Phylogenetic studies suggest that JE virus originated in Indonesia/Malaysia and spread from there into different parts of Asia with genotype IV being ancestral to the other genotypes. Genotype III was the dominant genotype in Asia until the mid-1990s and has been gradually replaced by genotype I over time, such that genotype I is now the dominant genotype. The mechanism of genotype replacement is not known at the present time.

Epidemiology

Distribution and Geography. JE is transmitted in nearly every country of Asia from the far eastern end of Russia to areas of Pakistan in west Asia (Fig. 1). Approximately 68,000 cases are reported annually from the region, including 13,600 to 20,400 deaths (55) principally from China, Southeast Asia, and India. In most areas, transmission is endemic with annual fluctuations in the number of cases, depending on environmental factors. Within a single country, transmission may be localized to certain regions where appropriate ecologic conditions prevail (in general, in rural areas where rice is grown). In developed countries such as Japan, South Korea, and Taiwan, the low incidence reflects high immunization rates despite the persistence of enzootic virus transmission.

Incidence and Prevalence. Estimates of the subclinical/clinical infection ratio usually center around 250:1, although values range from 25:1 to 1,000:1 (56). Exposure to JE virus occurs in childhood, and antibody prevalence approaches 80% by early adulthood. Annual incidence rates in locations of endemicity typically range from 1 to 10 overt cases/100,000, and epidemic attack rates may exceed 100/100,000. Case fatality rates of up to 30% have been reported, with neurologic or psychiatric sequelae in 20 to 30% of survivors. The estimated annual incidence is 5.4 per 100,000 and 0.6 per 100,000 in the 0 to 14- and ≥15-year-old age groups, respectively (55). Vaccination has reduced JE incidence in many countries. In Japan and other developed Asian countries, JE risk is highest in the elderly, reflecting high vaccine coverage of children and undefined biological risk factors associated with aging. In areas with overlapping religious and cultural affiliations (e.g., Malaysia and Indonesia), Muslims who eschew pork and do not keep pigs are at lower risk; conversely, Hindu Bali and principally Buddhist Chinese Sarawak have the highest reported JE activity in their respective countries. Household crowding, low socioeconomic status, and lack of air conditioning appear to be risk factors for acquiring JE.

In countries where JE is endemic, JE is mostly a disease of children and young adults due to the fact that most adults are already immune. Increased incidence in the elderly has been reported in several countries, presumably due to waning of seroprotective immunity. Prior antiflavivirus antibodies do not appear to protect against infection with JE virus but may decrease morbidity (57). Repeated clinical infections have not been reported, but subclinical reinfections are common in areas of endemicity and probably provide natural immunity and boosters to immunization.

Transmission. With the rare exception of laboratory-acquired cases, infection is transmitted only by bites of infected mosquitoes. In temperate regions, JE virus is

FIGURE 1 Geographical distribution of JE.

transmitted in the late summer and early fall after the virus has been amplified by vector mosquitoes and pigs, and, in some areas, by a preceding phase of amplification in birds (Fig. 2). Pigs play a central role in virus amplification because they develop high and sustained levels of viremia and because their high body temperature and large hairless body surface area attract thousands of mosquitoes nightly. The ubiquitous place of pigs as backyard or even cohabiting livestock animals in rural Asia has led to high levels of human exposure in rural villages and often in household premises. In developed countries (like Japan), the centralization of pig rearing and modernized agricultural methods have served to reduce JE virus transmission. On the other hand, deforestation and agricultural development, especially the creation of irrigation schemes, have contributed to an increase in JE and other mosquito-borne diseases in other areas (e.g., central Sri Lanka and the Terai of Nepal).

The principal JE vectors are *Culex* mosquitoes that use ground pools and especially rice paddies for their larval stages. *Culex tritaeniorhynchus* is the principal vector in most areas of Asia; other important vectors include *Culex annulus* in Taiwan and Hong Kong; *Culex vishnui*, *Culex gelidus*,

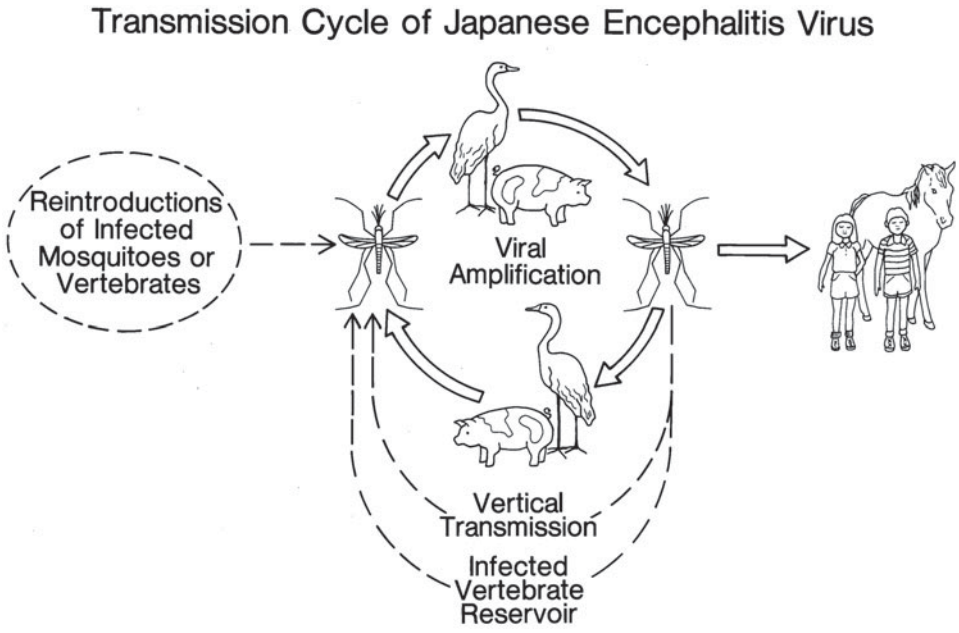

FIGURE 2 Transmission cycle of JE virus. Broken lines indicate speculative portions of the transmission cycle.

Culex pseudovishnui, *Culex fuscocephala*, *Culex bitaeniorhynchus*, and *Anopheles hyrcanus* in India and Nepal; *C. gelidus* in Indonesia; *Culex annulirostris* in the Pacific and Australia; and *Aedes togoi* in Russia. Most JE vectors are zoophilic, preferring animals to humans; exophilic, biting outdoors; and crepuscular, most active in the evening and night.

The transmission season is well defined in temperate areas (e.g., northern China), with onset of cases in June, a September peak, and disappearance by October. Farther south, the transmission season begins earlier and ends later. More complex seasonal patterns are observed in tropical areas where mosquito densities are correlated with monsoons. Vector density and infection rates typically increase following the initiation of rice cultivation midyear. Mosquito infection rates are modulated by rising herd immunity in pigs, but high vector abundance in the wet-cool season (October–December) ensures a continued risk of human infection.

Pathogenesis

The vast majority of infections are cleared before neuroinvasion occurs and are subclinical or lead to mild illnesses without CNS signs. Circulating antibody plays a critical part, and heterologous flavivirus immunity (e.g., from prior dengue virus infection) may limit peripheral virus replication and neuroinvasion. Disruption of the blood-brain barrier may be a risk factor for neuroinvasion, because persons with fatal JE are more likely to have had concurrent neurocysticercosis than persons dying of other causes; dual infections with herpes simplex virus or mumps virus also have been reported. Functional and structural changes due to hypertension, cerebrovascular disease, and head trauma have also been suggested as factors contributing to neuroinvasion.

At autopsy, inflammatory reactions are found in the myocardium, lungs, liver, spleen, lymph nodes, and kidneys. The brain appears swollen, and the ventricles may be narrowed by edema. Herniation of the cerebellar tonsils and hippocampal uncus may occur. The meninges are congested, and inflammatory changes are present. Pathological changes are distributed principally in the thalamus, substantia nigra, brain stem, hippocampus and temporal cortex, cerebellum, and spinal cord. Histopathology shows focal neuronal degeneration, diffuse and focal microglial proliferation, and perivascular cuffing (78). Infected neurons contain antigen in their cell bodies, axons, and dendrites, suggesting that virus spreads from cell to cell within the brain. Antigen-containing neurons may have no associated microglial reaction until cell death has occurred. Small amounts of virus antigen may be present in vascular endothelia.

Infection elicits a broad inflammatory response of macrophages, T and B cells in perivascular cuffs, and predominantly T cells in the brain parenchyma. A greater proportion of $CD4^+$ T cells are seen in the CNS than in the blood. Degeneration of infected neurons, microglial proliferation, and neuronophagia lead to the formation of gliomesenchymal nodules. Intrathecal antibodies have been associated with a favorable outcome in some series, suggesting an important role for antibody-mediated virus neutralization in the brain. However, others suggest that specific antibodies cannot reach virus spreading directly from cell to cell and that neuronal damage occurs by an immunopathological mechanism. This view is supported by observations of intrathecal neurofilament protein antibodies and myelin basic protein antibodies in 49% of cases and their association with fatal outcome (58). Immune complexes, found in the cerebrospinal fluid (CSF) of 17% of patients, are associated with death (35). Immune $CD4^+$ and $CD8^+$ T cells proliferate in response to JE virus or infected cell lysates, but cellular proliferative and humoral responses are not correlated, and no association between cellular proliferative response and outcome has been found (58). *In vivo* models indicate that activation of microglial cells may produce pro-inflammatory mediators instrumental in inducing neuronal cell death (59).

Clinical Manifestations

After a mosquito bite, 4 to 14 days elapse before the onset of symptoms. A prodromal illness of fever, headache, lethargy, nausea, and vomiting lasting several days precedes the onset of CNS signs. Neurologic signs reflect damage to the brain stem, thalamus, cerebral cortex, and spinal cord. The central feature is an altered state of consciousness, ranging from mild mental clouding to drowsiness and stupor, or agitation and delirium (60). Some children exhibit personality changes, slurred speech, and mutism. Early-onset seizures occur in at least half of hospitalized children and a quarter of adults. Seizures are usually generalized tonic-clonic but may also be partial motor or with more subtle clinical manifestations, such as twitching of a digit or eyebrow or nystagmus. Patients with subtle seizures are usually in status epilepticus. Extrapyramidal features include dull, expressionless faces, generalized hypertonia, and cogwheel rigidity.

Significant stupor or coma is usual, tremor and involuntary movements are common, and signs of meningeal irritation may be present. Disconjugate gaze and facial and other cranial nerve palsies are found in one-third of cases. Papilledema is seen in fewer than 10% of cases. The muscular tone is usually increased, and hyperreflexia and pathological reflexes may be elicited. Weakness or paralysis may be generalized or asymmetric in distribution and spastic or flaccid in character. Autonomic disturbances, especially hyperpnea, may occur.

A modest peripheral leukocytosis may be present in the first week of illness. The serum sodium often is depressed due to inappropriate ADH secretion. The CSF typically is clear and under normal pressure, with normal or mildly elevated protein and normal glucose concentrations. A pleocytosis of 10 to several hundred white cells is typical in the first week of illness, and the cell count may remain elevated until the third week. EEG tracings show generalized delta-wave slowing, consistent with a thalamic involvement. Computed tomography (CT) scans show diffuse white matter edema and nonenhancing low-density lesions mainly in the thalamus, basal ganglia, and pons. Unilateral or bilateral thalamic hemorrhages are common. Magnetic resonance imaging (MRI) discloses a similar pattern of high-signal-intensity lesions in the thalamus, basal ganglia, cerebellum, pons, midbrain, and spinal cord.

Defervescence occurs during the second week of illness; choreoathetosis and extrapyramidal signs may appear as other neurologic manifestations improve. The substantia nigra is a site of predominant injury in some patients, as detected by MRI (61, 62) and clinical presentation, with cogwheel rigidity and opsoclonus during the acute phase and emergence of typical Parkinsonism during convalescence, from which patients generally recover (63). The illness is fatal in 10 to 35% of cases, most often within the first week, and fatality rates have been decreasing with improved clinical management. Children younger than 10 years are more likely to die and to have serious neurologic sequelae, such as motor weakness and paralysis, abnormal muscular

tone, seizures, blindness, ataxia, and extrapyramidal movement disorders (64). Convalescence is slow; consciousness and motor functions are gradually regained. One-third to one-half of patients surviving the acute illness have such major neurologic sequelae 1 year later, and EEG abnormalities may be present in 50% of recovered children. At 5 years after recovery, 75% have behavioral disorders and subnormal performance on age-standardized psychological tests.

JE acquired during the first and second trimesters of pregnancy has led to fetal death and abortion, with recovery of virus isolates from products of conception (65). It is unknown whether congenital infection causes fetal malformations, as occurs in pigs. The few reported cases of JE in the third trimester have been associated with a normal fetal outcome.

The most common complications are bacterial infections, especially pneumonia, and stasis ulcers. In a few cases, clinical relapse occurred several months after recovery from the acute illness. JE virus–infected peripheral-blood mononuclear cells were demonstrated in these and other asymptomatic recovered patients, implying the persistence of infection. Evidence of subacute JE virus CNS infection has been demonstrated in 5% of cases by detecting JE virus or antigen in CSF 3 weeks after recovery or intrathecal IgM at 50 to 180 days. The clinical significance of these findings is unclear.

Diagnosis

In Asia, the most important diagnostic considerations in children with signs of acute CNS infection are bacterial meningitis, tuberculosis, malaria, typhoid fever with associated tremors and ataxia, dengue disease with encephalopathy or encephalitis, WNV encephalitis, MVE (in Australia and Papua New Guinea), Nipah virus encephalitis, and enterovirus (particularly enterovirus 71) infection. Other disorders that overlap the clinical presentation of JE are hyperthermia and scrub typhus. JE occasionally may present as Guillain-Barré syndrome (GBS) or acute psychosis. In Asia, the percentage of children presenting with acute encephalitic illness, who had JE, ranged from 23 to 67%. Central hyperpnea and extrapyramidal signs were the best clinical predictors (sensitivity of 41% and specificity of 81%) that the illness was JE (66).

JE virus rarely can be isolated from blood early in the illness, usually no later than 6 to 7 days after infection. Virus is seldom recovered from the CSF except in cases with a poor outcome. RT-PCR of CSF specimens is not a more sensitive technique. Immunofluorescence (IF) staining of CSF mononuclear cells provides laboratory confirmation most rapidly but has a reported sensitivity of only 60% (67).

The standard laboratory diagnostic method is serology by IgM capture ELISA, which has a sensitivity approaching 100% when both CSF and serum samples are tested and samples from 1 to 2 weeks after onset are used. An evaluation of three commercially available IgM capture ELISA kits demonstrated sensitivities of at least 89%; however, specificities ranged from 56 to 99% (68). Specificities were at least 96% when dengue virus IgM-positive samples were excluded. Subsequently, two IgM capture ELISAs were field tested with reasonable sensitivities and excellent specificities and little cross-reaction with dengue (69, 70). Samples taken too early in the illness may be negative. Anti-JE virus IgM was detected in nearly all serum samples collected at least 9 days after symptom onset and in all CSF samples collected at least 7 days after symptom onset (26). This and all serologic tests are limited by the potential for cross-reactions with cocirculating flaviviruses, principally dengue virus and WNV. Representative flavivirus antigens relevant to the patient's site of exposure should be included in serologic tests. Some laboratories have established diagnostic IgM ELISA absorbance ratios based on comparative reactivities to the respective antigens. Fourfold changes in IF, hemagglutination-inhibiting (HI), CF, and neutralizing antibodies (in order of increasing specificity) may provide a clear diagnosis in patients with their first flavivirus infection, but cross-reactions may be uninterpretable for patients with multiple previous flavivirus infections or immunization with JE and yellow fever vaccines.

Prevention

In areas of Asia where JE is endemic, childhood vaccination has led to significant declines in disease incidence, and in some countries the near disappearance of the disease. Approximately half of the countries where JE is endemic have routine immunization programs. Formalin-inactivated, mouse-brain-derived vaccine is still produced in a few countries, but manufacture is being phased out in place of Vero-cell-culture-derived inactivated vaccines. The Vero-cell-culture-derived vaccine (IXIARO) was introduced into the United States in 2009 and replaced the mouse-brain-derived vaccine manufactured by BIKEN (Osaka, Japan; licensed as JE-VAX) in 2012. The vaccine is licensed for use in those aged >2 months in the US (and >6 months of age in endemic countries). The vaccine is supplied as a two-dose primary immunization series, given on 0 and 28 days, with children aged 2 months to ≤3 years getting half of the dose used in those 3 years or older. Vaccinees are not to visit JE endemic areas until at least 7 days after receiving the second dose of vaccine. It is approved for HIV-infected and immunocompromised individuals, although the immune response induced may be less than that in healthy individuals. An accelerated 0- to 7-day immunization regimen is noninferior to the standard 0-, 28-day regimen (71).

A live, attenuated vaccine (SA14-14-2) produced in primary hamster kidney cell culture was licensed in China in 1988, and more than 350 million doses have been administered as a single dose vaccine in those aged 8 months or older. The vaccine and was shown to be 96% effective with no evidence of reactogenicity in Nepalese children 5 years after immunization (72). In addition, there is a live, attenuated recombinant-chimeric yellow fever/JE vaccine based on the yellow fever 17D vaccine virus backbone with the structural protein genes (premembrane and envelope protein genes) of SA14-14-2 virus (73). This vaccine has been licensed as a single-dose vaccine for those aged 9 months and older in Australia and some Asian countries.

Exposure to JE mosquito vectors can be reduced by avoiding rural areas and outdoor activity during the evening, staying in air-conditioned or well-screened quarters, using repellents, and wearing long-sleeved clothing and long pants. Specific public health interventions to control vector mosquitoes are implemented in a few locations. These include the addition of larvicides and predatory fish to irrigated fields and periodically changing their water level to interfere with the vectors' aquatic stages. In epidemic emergencies, adulticide applications provide short-term reductions of vector mosquito populations.

Treatment

No specific antiviral therapy is currently available, and there is little published on candidate antivirals. In a double-blind, placebo-controlled trial, IFN-*a*2a did not improve the outcome of patients with JE (74). The introduction of

intensive-care equipment and training has reduced the mortality rate from more than 30 to 5%. Acute coma, seizures, and respiratory failure necessitate rapid anticonvulsive therapy, ventilatory support, and intensive monitoring of fluid and electrolyte balance. Osmotic agents to reduce intracranial pressure may be needed in some cases; a controlled study of early dexamethasone therapy found no clinical benefit (or harm) with its routine use (75). Hyponatremia due to inappropriate ADH secretion is a frequent complication usually responsive to free water restriction alone.

Murray Valley Encephalitis Virus

Biology

MVE virus is a member of the JE antigenic complex and has a close genetic relationship to JE virus. Australian MVE virus strains share a high degree of sequence homogeneity, whereas strains from Papua New Guinea differ, indicating that this area may represent a separate focus of virus activity. The host range of MVE virus and susceptibility of cell cultures are shown in Table 3. Alfuy virus, also found in Australia, is a distinct species from MVE virus (76).

Epidemiology

Distribution and Incidence. MVE virus occurs in Australia, Papua New Guinea, and probably islands in the eastern part of the Indonesian archipelago (77). Localized seasonal outbreaks or sporadic cases have been reported intermittently from southeastern Australia, the Kimberley region in Western Australia, and the Northern Territory. Although the disease is clinically severe, incidence has been low. The last major outbreak was in 1974 involving 58 cases over a wide geographic area, including Queensland, Northern Territory, South Australia, the Ord River Basin of Western Australia, and Papua New Guinea. In 2011, the largest outbreak since 1974 caused 17 cases (including 3 deaths). Significantly, half of the cases did not live in areas where they were infected (78).

MVE primarily strikes the young and the elderly, with approximately 50% of the cases in infants and children under 10 years old and 25% in persons over 50 years old. An excess of cases among males has been observed. The case fatality rate is 15 to 30% and long-term sequelae in 30 to 50%; only 40% have complete recovery. Subclinical infections are frequent, and it is thought that only one in 150 to 1,000 infections results in clinical illness. Serologic surveys conducted after epidemics have revealed evidence of recent infection in 4.5 to 36% of the residents. Attempts to associate MVE virus with mild febrile illnesses without neurologic signs have not been successful.

Virus circulation is seasonal, occurring during the summer months (January to May), and is associated with periods of abnormally high rainfall over two consecutive years. Ecological factors influencing vector density, such as the development of irrigation projects, have been linked to the emergence of MVE epidemics.

Transmission. The primary summertime transmission cycle involves wild avian hosts and *Culex annulirostris* mosquitoes, a species that breeds in temporary pools. Aquatic birds, such as herons, egrets, and pelicans, serve as the major viremic hosts, but mammals (especially rabbits and possibly kangaroos) may also play a role in transmission. *Culex annulirostris* has been implicated in tangential transmission of MVE virus to humans, which are dead-end hosts. No human-to-human spread is recognized. The overwintering mechanism and the factors responsible for emergence of intermittent epidemics are unknown.

Pathogenesis

Like other members of the JE virus complex, MVE virus is highly neurotropic. The virus has been recovered from human brain tissue obtained postmortem. Pathological findings are similar to those in JE (79) and are limited to the CNS.

HI and neutralizing antibodies appear during the first week after onset. IgM antibodies appear early and may persist for weeks. Immunity to reinfection is believed to be lifelong. The presence of closely related flaviviruses in Australia, including Kunjin virus (WNV) and JE, dengue, and Kokobera viruses, complicates serodiagnosis.

Clinical Manifestations

The incubation period is 1 to 4 weeks. The clinical features of MVE (80) include a 2- to 5-day nonspecific illness, which precedes CNS involvement, with sudden onset of fever, headache, myalgia, generalized malaise, anorexia, and nausea. This is followed by the onset of nuchal rigidity and neurologic signs. In infants the disease progresses rapidly, and coma and death may occur within 24 hours of onset of neurologic signs, often with clinical signs of involvement of the brain stem and spinal cord. The clinical features are classified by illness severity (81): (i) mild cases with altered levels of consciousness and variable neurologic abnormalities, but without coma or respiratory depression, and stabilization of neurologic signs within 5 to 10 days; (ii) severe cases with seizures, coma, and paralysis, often requiring respiratory assistance; and (iii) fatal cases with spastic quadriplegia and progressive CNS damage or severe disease with superimposed bacterial infection. Seizures and flaccid paralysis are rare in adult patients. The CSF shows pleocytosis, which shifts from polymorphonuclear to mononuclear predominance after a few days of onset; normal or mildly elevated protein; and normal glucose concentration. Neurologic sequelae occur in up to 40% of the mild cases and in all of the severe cases from which patients recover. Abnormalities include cerebral atrophy, paraplegia, impaired gait and motor coordination, and intellectual deterioration. Recovery or death generally occurs within 2 weeks of onset. Respiratory failure, intercurrent bacterial infections, and the residual neuropsychiatric deficits represent the most important complications of MVE.

Diagnosis

Viremia is present early in infection, but virus isolation or viral nucleic acid detection from blood (or CSF) has not generally been successful. Serologic diagnosis is based on ELISA or HI, CF, or neutralization tests. IgM antibodies appear to be quite specific and useful for early diagnosis, the test becoming positive around 4 to 9 days after onset and can persist for months. Cross-reactions with Kunjin virus and other flaviviruses may confuse interpretation. An epitope-blocking ELISA with MVE virus monoclonal antibodies distinguished MVE virus infections from those with other flaviviruses. Postmortem diagnosis is possible by examination of brain tissue by virus culture, direct detection of the virus genome by RT-PCR, or detection of virus antigen by immunohistochemistry.

Prevention

No vaccine is available. It is unknown whether active immunization with JE vaccine would provide a degree of

cross-protection against MVE. However, mice immunized with inactivated mouse-brain-derived JE vaccine and challenged with MVE were not protected, and, in fact, had accelerated deaths, consistent with an immunopathological process. In contrast, the live chimeric JE vaccines, ChimeriVax-JE [yellow fever 17D vaccine virus backbone with JE premembrane and envelope protein genes], gave complete and durable (>5 months) protective immunity against MVE virus challenge (82). In areas prone to recurrent epidemics (e.g., the Murray-Darling River basin), reduction of *Culex annulirostris* breeding by use of larvicides is practiced. Treatment of MVE is symptomatic.

Treatment

Treatment of MVE is symptomatic and consists of good general management and nursing care, especially in the semicomatose and comatose patient, and respiratory support as required. No effective antiviral chemotherapeutic agent is available (80). Neither IFN nor ribavirin has been studied.

Powassan Virus

Powassan virus is related to the eastern hemisphere's TBE viruses and is maintained in a cycle between ticks and rodents (83). Two lineages of Powassan virus exist in North America. Lineage 1 Powassan virus is associated with *Ixodes cookie* ticks and woodchucks and *Ixodes marxi*, ticks and squirrels. These ticks rarely bite humans. Lineage 2 Powassan virus, sometimes called deer tick virus, is associated with *Ixodes scapularis* ticks, the same tick that transmits Lyme disease, babesiosis, and anaplasmosis.

Since its discovery in 1958, Powassan virus has become recognized as an uncommon cause of encephalitis in Russia, eastern Canada, and the north central, northeastern, and upper Midwestern United States. The incidence of Powassan virus infection appears to be increasing from 2004 through 2013, during which 57 cases were reported to the United States Centers for Disease Control and Prevention. Infection mostly occurs from June to September.

The reported incubation periods range from 8 to 34 days (84, 85). However, patients infrequently recall a tick bite, because ixodid ticks are small and can be easily overlooked. Symptomatic patients typically present with fever, weakness, paralysis, somnolence, gastrointestinal complaints, headache, and confusion; seizures can occur. The case-fatality rate is 5 to 10 percent, with a high incidence of residual neurological dysfunction among survivors, including hemiplegia, headaches, minor memory impairment, and persistent ophthalmoplegia (84–86).

The diagnosis of Powassan virus infection can be made by demonstration of IgM antibody by capture immunoassay of CSF, a fourfold rise in serum antibody titers against the virus, or isolation of virus from or demonstration of viral antigen or nucleotide sequences in tissue, blood, or CSF. IgM antibodies alone should be confirmed by demonstration of IgG antibody by another serologic assay (e.g., neutralization).

No specific vaccine or antiviral treatment is available.

Flaviviruses associated with rare and sporadic cases of human disease are shown in Table 4.

Saint Louis Encephalitis Virus

Biology

Saint Louis encephalitis (SLE) virus is a member of the JE antigenic complex. Antigenic cross reactivities between SLE and other members of the complex are demonstrable with polyclonal and monoclonal antibodies. All SLE strains belong to a single serotype defined by neutralization, but strain variation occurs at the molecular level. The nucleotide sequence of the SLE E gene shows <10% nucleotide difference among strains spanning a >60-year interval (87, 88). Genomic conservation suggests that the virus is adapted to a highly constrained system of virus maintenance in vectors and vertebrate hosts. SLE virus strains from North America and from Central and South America can be divided into seven lineages, which are predominantly, but not strictly, geographically distinct (88).

Among commonly used laboratory animals, hamsters are most susceptible to lethal infection, followed by mice (Table 3). Young animals are most susceptible to lethal encephalitis. Neurovirulence is greater with epidemic, than with endemic, strains. SLE virus is lethal for chick embryos or after intracerebral inoculation of young hatchling birds, but birds infected naturally develop asymptomatic viremic infections. Birds are the principal hosts, amplifying virus in the transmission cycle. The absence of overt disease associated with SLE infection of birds is due to a long co-evolution of virus strains with their hosts in the Americas. Although various wild and domestic mammals can be infected with SLE virus, these infections are also usually asymptomatic. Antibodies have been found in a wide array of wild mammals in North and South America. Additional information on host range and cell culture susceptibility is given in Table 3.

Epidemiology

Distribution and Geography. SLE virus is widely distributed in the Americas. Although cases have been reported from nearly all US states, incidence is highest in states in the Ohio-Mississippi River basins and on the Gulf Coast (Fig. 3). The geography of SLE in the United States can best be understood in terms of its three enzootic transmission cycles sustained by *Culex pipiens* and *Cx. quinquefasciatus* in the Ohio and Mississippi river basin states and areas farther east; *Cx. nigripalpus* in Florida; and *Cx. tarsalis* in the central and western states, west of, and including, Minnesota, Iowa, Kansas, Oklahoma, and west Texas (Fig. 4). In the east, SLE is transmitted periodically in localized, and sometimes regional, outbreaks occurring at intervals of years and even decades apart but without significant enzootic or endemic transmission in intervening years.

Incidence and Prevalence. More than 10,000 SLE cases have been reported in the United States over the last five decades, although only 1 to 12 cases per year were reported from 2004 to 2013, most likely due to competition for the same mosquito vectors by West Nile virus (Fig. 5). However, a concurrent outbreak of SLE and West Nile virus occurred in Phoenix, Arizona, in 2015 (89). Most cases have occurred as sporadic outbreaks, with the largest resulting in 1,967 reported cases from 31 states in 1975. Population seroprevalence rates are generally low, with seroprevalence ranging from 3 to 15% following outbreaks. Only 1 of approximately 300 infections is clinically apparent. Although infections are uniform across the age spectrum, clinical attack rates rise steeply with age, resulting in inapparent/apparent infection ratios ranging from 800:1 in children younger than 10 years to 85:1 in adults.

SLE in eastern states follows an unpredictable pattern of localized outbreaks, usually in urban areas (90). The principal vectors, *Cx. pipiens* and *Cx. quinquefasciatus*, are typically found in peridomestic habitats. Adult mosquitoes

TABLE 4 Flaviviruses causing sporadic human disease[a]

Virus	Human disease			Veterinary disease species affected	Geographic distribution	Transmission cycle
	No. of cases	Clinical features	Severity			
Banzi virus	< 10	Nonspecific febrile illness	Self-limited	None known	Southern and East Africa	Mosquitoes (*Culex*)-rodents
Bussuquara virus	1	Fever, arthralgia	Self-limited	None known	Brazil, Colombia, Panama	Mosquitoes (*Culex*)-rodents
Edge Hill virus	1	Fever, myalgia, arthralgia	Self-limited	None known	Australia	Mosquitoes (*Aedes*)-wallabies
Ilheus virus	6	Fever, myalgia, encephalitis[b]	Potentially severe (no deaths)	None known	Brazil, Colombia, Panama, Trinidad	Mosquitoes (*Psorophora*)-birds
Kokobera virus	3	Fever, rash, arthralgia	Self-limited	None known	Australia, Papua New Guinea	Mosquitoes (*Culex*)-?
Koutango virus	1[c]	Fever, rash, arthralgia	Self-limited	None known	West Central Africa	Ticks (several genera)-rodents
Kunjin virus	< 20	Fever, rash, encephalitis	Potentially severe (no deaths)	Horses (rare)[e]	Australia, Sarawak, Thailand	Mosquitoes (*Culex*)-birds
Langat virus	< 10	Fever, encephalitis[b]	Potentially severe (no deaths)	None known	Malaysia, Thailand, Russia	Ticks (*Ixodes*)-rodents
Louping ill virus	39[d]	Similar to CEE[f]	Potentially severe (no deaths)	Sheep[g]	United Kingdom, Ireland	Ticks (*Ixodes*)-sheep, grouse
Modoc virus	1	Aseptic meningitis	Self-limited	None known	Western United States, Canada	Rodent-rodent[h]
Negishi virus	3+[i]	Encephalitis	Potentially fatal	None known	Japan, China	Ticks-?
Rio Bravo virus	6 (11)	Febrile illness, meningitis, orchitis	Self-limited	None known	Western United States, Mexico	Bat-bat
Sepik virus	1	Febrile illness (hospitalized)	Self-limited	None known	New Guinea	Mosquitoes (*Mansonia*)-?
Spondweni virus	3	Fever, arthralgia, rash	Self-limited	None known	South and West Africa	Mosquitoes (*Aedes*)
Usutu virus	1	Fever, rash	Self-limited	None known	South and Central Africa	Mosquitoes (*Culex*)-birds
Wesselsbron virus	< 20	Fever, arthralgia, rash, encephalitis	Potentially severe (no deaths)	Sheep[h]	Sub-Saharan Africa, Thailand	Mosquitoes (*Aedes*)-?

[a]The recognition of these diseases has often occurred in the setting of general virus investigations and surveillance projects. Infection (disease) may be more common than indicated, and the clinical spectrum may differ from that delineated by the few recognized cases.
[b]Includes cancer patients intentionally infected who developed encephalitis.
[c]Laboratory infection.
[d]26 cases were laboratory infections.
[e]Encephalomyelitis.
[f]In one case a hemorrhagic diathesis was described.
[g]Encephalitis (cerebellar syndrome). Sporadic disease also described in dogs, horses, cows, goats, pigs, and deer.
[h]Virus without known intermediate arthropod vector; transmission by contact or aerosol.
[i]Includes a laboratory infection.

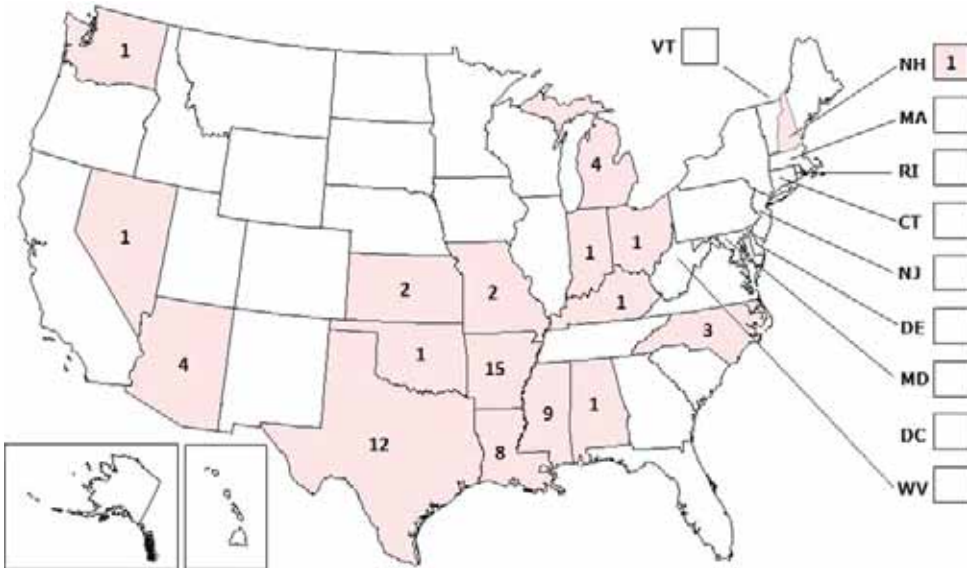

FIGURE 3 SLE neuroinvasive disease cases by state, 2004–2013.

use open house foundations and urban storm sewers as resting sites, and polluted ground water is available for their larval stages. Outbreaks often have occurred in small towns or in older lower socioeconomic sections of cities. Older construction, featuring open foundations, porches, lack of air conditioning, and poor screens, contributes to an increased risk of exposure. Local or regional outbreaks occur infrequently, sometimes separated by decades when, for unknown reasons, virus transmission in the enzootic cycle becomes sufficiently intense to create substantial human infection risk. The overwintering mechanism for SLE virus remains unclear.

SLE transmission in Florida has followed a pattern of periodic focal or widespread outbreaks, but the disease may be absent. Drought conditions might concentrate C*x. nigripalpus* mosquitoes in relatively moist, densely vegetated "hammock" habitats at a time when nesting birds also make use of the hammocks (91). This forcing of birds and mosquitoes together fosters epizootic SLE amplification. During subsequent wet periods, infected birds and mosquitoes disperse and carry the virus from the hammocks.

In the west, SLE is transmitted perennially and at a low level in the enzootic transmission cycle; this results in sporadic human infections, a higher level of naturally acquired immunity, and, occasionally, outbreaks that are usually smaller than those occurring in the east.

SLE cases are usually noted no earlier than June and peak in August and September. Transmission may continue as late as December/January in Florida and in parts of southern California. Elevated temperatures enhance multiplication of SLE virus in C*x. tarsalis*. Rainfall patterns also markedly influence SLE virus transmission. Years of high spring and summer rainfall or excessive runoff from snowmelt favor vector density, and virus amplification in areas where C*x. nigripalpus* and C*x. tarsalis* are responsible for transmission. In contrast, mid-summer drought conditions appear to favor amplification in the C*x. pipiens*-C*x. quinquefasciatus* transmission cycle, because these vectors breed in stagnant water pools susceptible to being flushed and cleaned by rainfall.

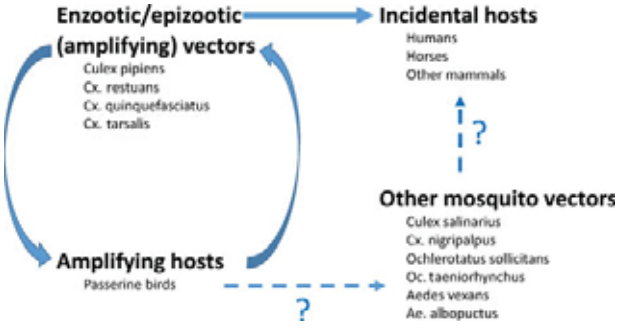

FIGURE 4 Transmission cycle of SLE virus and WNV. Both viruses are transmitted in a cycle between birds and Culex mosquitoes. C. *pipiens* is an important vector in the northern United States and Canada. *Culex quinquefasciatus* is important in the southern United States, whereas C. *tarsalis* is important in the western United States. In addition, C. *nigripalpus* is a vector in Florida. Other mosquito species may be "bridge" vectors to horses, humans, and other dead-end hosts, which typically do not develop high-level viremia and do not participate in the transmission cycle. However, bridge vectors likely have a minor role compared to enzootic vectors in viral transmission to dead-end hosts.

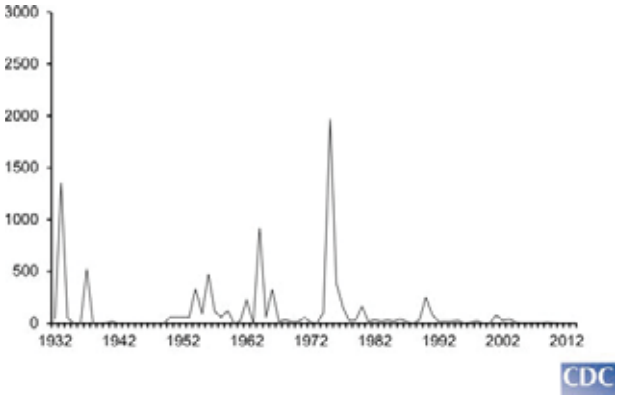

FIGURE 5 Number of reported cases of SLE, 1932–2012.

Risk Factors Associated with Human Disease. Risk of exposure and infection are independent of age, but clinical attack rates and severity of illness, as measured by case-fatality ratio, all rise sharply with age. Age-specific rates are approximately 10-fold higher in persons older than 70 years than in young adults and children. Hypertension, arteriosclerosis, and other chronic diseases have been noted in fatal cases. Although such associations with symptomatic illness have not been controlled for age, they suggest that virus neuroinvasion might be facilitated through compromised vascular endothelia.

The principal risk factor for *Cx. tarsalis*–borne SLE is residence in, or outdoor occupational or avocational exposure to, rural areas where the enzootic cycle is maintained. Risk is greatest in persons who are outdoors in the evening when *Cx. tarsalis* is most active. In Florida, a similar pattern holds because *Cx. nigripalpus* has analogous exophilic crepuscular biting habits. In some *Cx. tarsalis*–borne outbreaks, risk has been higher in males, possibly reflecting occupational or avocational exposures. The peridomestic habits of SLE vectors (*Cx. quinquefasciatus* and *Cx. pipiens)* elsewhere in the eastern states have been associated with higher incidence in females in some outbreaks, although this pattern is not consistent. Risk factors, such as lack of air conditioning, poor screens, and sitting on an unscreened porch, suggest that exposure occurs indoors or near residences. In some urban outbreaks, a large proportion of cases have occurred in homeless people.

Transmission. Natural infections are transmitted by mosquito bites. Laboratory infection occurred by mucous membrane or respiratory exposure in one reported case. SLE virus antigen has been recovered from nasal secretions of infected mice, and hamsters can be experimentally infected by intranasal inoculation, suggesting the potential for infection via the olfactory apparatus. No nosocomial cases or instances of congenital infection have been reported. One possible transmission via breast milk has been reported.

Pathogenesis

Pathological changes in humans have been reported only for the CNS; however, SLE virus has been isolated from the vitreous humor, lungs, liver, spleen, kidneys, and blood. In fatal cases, the brain and meninges appear to be congested, but cerebral edema is unusual. Pathological changes are widely distributed in the brain and spinal cord but are most pronounced in the substantia nigra, thalamus, hypothalamus, cerebellar and cerebral cortices, basal ganglia, and the brain stem and cervical spinal cord. Changes are most prevalent in gray matter nuclei, but lesions also are found in the subcortical and deep cerebral white matter and long tracts of the brain stem and spinal cord. Microscopic changes consist of diffuse parenchymal inflammation and substantial perivascular infiltrates with destruction of vessels and extensive microgliosis. The inflammatory infiltrate is a mixture of small lymphocytes, histiocytes, plasma cells, and rare neutrophils. Degenerating neurons may be surrounded by microglial nodules. Axonal destruction and demyelination are not usually present. SLE virions have been identified by electron microscopy and SLE antigen by immunohistochemistry in postmortem brain samples.

Clinical Manifestations

The incubation period has been estimated to be 4 to 21 days. Illness begins with nonspecific symptoms of malaise, fever, headache, and myalgias, sometimes accompanied by cough, sore throat, abdominal pain, vomiting, or diarrhea. Dysuria, urgency, and incontinence are early symptoms in some patients. The illness may evolve quickly, with development of meningismus, increased headache, tremulousness, ataxia, and mental clouding. Other patients have a more insidious progression of nonspecific symptoms, especially myalgias, backache, headache, photophobia, and gastrointestinal complaints, followed days later by neurologic symptoms.

Neurologic involvement most frequently results in an altered state of consciousness, which may be clinically subtle, manifested by only mild confusion or decreased cognitive facility. Deep coma occurs in about 15% of cases. Most patients exhibit tremors involving the eyelids, tongue, lips, and extremities. Cranial nerve palsies are present in about 25% and are often asymmetrical or unilateral. Cerebellar signs are also common; ambulatory patients may exhibit an ataxic gait. Involuntary movements, such as myoclonic jerking, nystagmus, and, rarely, opsoclonus, may be present. Convulsions are unusual, occurring mainly in children and, in severe cases, in adults and signal a poor prognosis. Nuchal rigidity is variable and may be more common in children. Presentation with focal weakness or paralysis is unusual. Occasional cases have presented with Guillain-Barré syndrome.

The peripheral white cell count is usually normal or slightly elevated. Proteinuria, microscopic hematuria, and pyuria are found in some patients. More than one-third of cases have hyponatremia due to inappropriate antidiuretic hormone (ADH) secretion. Transaminases may be slightly elevated. Lumbar puncture discloses an elevated opening pressure in the range of 200 to 250 mmHg in about one-third of cases and CSF protein elevated above 45 mg/dL in more than two-thirds of cases. A moderate CSF pleocytosis of five to several hundred cells is typical, with the proportion of mononuclear cells increasing from 50 to 100% during the first week of illness. Electroencephalograms (EEGs) show diffuse generalized slowing and delta-wave activity. Focal discharges and spike activity have been observed occasionally. Neuroimaging may show hyperintensity of the substantia nigra on T2-weighted images.

Defervescence and neurologic improvement are seen over several days, with a rapid reversal of altered sensorium. Overall, 17% of cases are fatal; deaths generally occur within the first or second week of illness. A prolonged convalescence is typical, characterized principally by emotional lability but also by asthenia, forgetfulness, headache, tremor, dizziness, and unsteadiness; such manifestations may persist for months to years after initial recovery. Muscle tremors, asymmetric deep tendon reflexes, and visual disturbances are present in about one-third of patients several months after the acute illness. One patient had postinfectious encephalomyelitis; otherwise, chronic, persistent, or relapsing infections have not been reported.

Diagnosis

SLE should be considered in patients with onset of acute encephalitis in summer or early fall and with exposure to an endemic or epidemic focus. Clinical features may overlap those of other virus infections, especially WNV, enteroviruses, western and eastern equine encephalitis, and herpesvirus. Mycoplasmal, bacterial, and fungal meningitis, especially partially treated bacterial meningitis, leptospirosis, cat scratch encephalopathy, and listeria encephalitis, may cause a CNS syndrome similar to SLE.

The limited viremia preceding onset of neurologic symptoms precludes virus isolation in most cases with

clinical encephalitis. Virus isolation from CSF is generally unsuccessful, but virus can be recovered from brain and other postmortem tissues. Viral antigen can be detected by IF or immunocytochemical staining in brain tissue, but the sensitivity of this technique is low because of the sparse distribution of infected cells. Antigen detection ELISAs and RT-PCR developed to identify infected mosquitoes have not been evaluated on clinical samples, but they are likely to be less sensitive than serology.

The principal serologic tests in use are IgM capture ELISA and IF. When both CSF and serum samples are examined, sensitivity of the former test approaches 100% by the 10th day after onset. The sensitivity of indirect IF is lower, especially when CSF is examined or if only acute-phase serum is examined. When both IgG and IgM are measured by indirect IF or ELISA in paired serum samples, sensitivity approaches 100%. Cross-reactive antibodies in patients who have had previous flavivirus infections, especially dengue virus and WNV, limit the specificity of serologic tests. More specific serologic tests (e.g., neutralization) are needed to sort out such reactions. Viral RNA detection is usually used for blood donations and transfusions.

Prevention

Efforts to prevent SLE epidemics are based on mosquito- and bird- or sentinel-chicken surveillance that measures enzootic virus activity in the spring and summer. Early increased transmission in the enzootic cycle signals the possibility of spillover to humans and should trigger emergency mosquito control with insecticides and public health warnings to avoid mosquito exposure. Reduction of larval habitat by improving drainage is a long-term approach to reduce risk. Repairing screens, avoiding outdoor exposure in the evening, and applying mosquito repellents may reduce mosquito exposure. No vaccine is currently available.

Treatment

No specific antiviral therapy is currently available. As with other flaviviruses, ribavirin is active *in vitro* at relatively high concentrations, but there is no evidence for its clinical efficacy. A nonrandomized, unblinded clinical trial suggested a possible benefit of IFN-*a*2b treatment in patients with neuroinvasive disease (92).

Tick-borne Encephalitis Virus (CEE and RSSE)

Russian spring-summer encephalitis (RSSE) virus was isolated from human brain tissue in the far east of the former Soviet Union in 1937. A similar disease was first described in Czechoslovakia in 1948, and the causative virus (Central European encephalitis [CEE] virus) was shown to be serologically related to RSSE virus. These agents, transmitted by the bite of small ixodid ticks, cause severe neurological infections and represent an important health threat over large areas of Europe and Asia.

Biology

Originally the TBEV virus complex contained 14 antigenically-related agents, 8 of which were human pathogens (Table 1). Genetic studies have resulted in the TBEV complex being divided into two complexes: mammalian tick-borne virus group and seabird tick-borne virus group. Within the mammalian tick-borne virus group are nine distinct species: Gadgets Gully, Kadam, Kyasanur Forest disease, Langat, Louping ill, Omsk hemorrhagic fever, Powassan, Royal Farm, and TBEV (3). TBEV has three subtypes: central European encephalitis (CEE; also called western subtype), Russian spring-summer encephalitis (RSSE; also called far eastern subtype), and Siberian (also called Vasilchenko virus) (93, 94). Louping ill has at least five subtypes (British, Greek, Irish, Spanish, and Turkish), and Kyasanur Forest disease virus has Alkhumra virus as a subtype. The close antigenic and genetic relationships of these viruses create difficulty in identifying individual virus species, and, hence, much debate. Indeed, debate remains whether the Turkish subtype is a distinct species. Nonetheless, immunity to CEE and RSSE is cross-protective. Within a subtype, strains are relatively homogeneous, varying by a maximum of 2.2% nucleotide variation, while a maximum difference between subtypes is 5.6%. It is proposed that the TBEVs evolved approximately 2,000 years ago (5). Evolution of this group of viruses is characterized by the emergence of a small number of stable lineages, reflecting the slow rate of transmission of these agents and restricted movements of vectors and terrestrial (rodent) hosts.

The host range and cell culture susceptibility of tick-borne flaviviruses vary substantially (Table 3). Within a subtype, virus strains may vary considerably with respect to virulence (95). Single amino acid changes in the E gene have been shown to alter the virulence of CEE virus (10). Most domestic livestock (cows, goats, and sheep) develop viremia and secrete virus in their milk but do not develop clinical illness. Dogs are susceptible to encephalitis acquired by tick bite.

Epidemiology

Distribution and incidence. TBE occurs in an endemic pattern over a wide area of Russia and the independent states of the former Soviet Union, Central and Eastern Europe, and Scandinavia, Switzerland, Germany, Italy, Greece, Albania, China, and Japan (Fig. 6). Austria, the Czech Republic, Slovakia, Slovenia, and Hungary experience the highest incidences, with several hundred cases each year and morbidity rates between 1 and 20 per 100,000. In Russia, 3,000 to 6,000 cases occur annually, and the highest incidence is in western Siberia, with morbidity rates of 5 to 10 per 100,000. In Japan, sporadic cases of RSSE occur in Hokkaido. Among American military personnel and travelers in Germany, the risk of infection was found to be approximately 1 per 1,000 person-months of exposure (96).

TBEV has relatively constant distribution coinciding with ecologic habitats favorable for tick activity. TBEV is usually found in valleys and nearby rivers with adequate habitats for ticks; however, in recent years, TBEV has also been reported at altitudes up to 2,100 m above sea level (97). The risk of human exposure to ticks largely depends on outdoor activities. Occupations (forestry and farming), mushroom- and berry-picking, and recreational activities in forested and scrub areas are risk factors for infection. Approximately two-thirds of the cases occur in young adults (20 to 40 years of age), and males are at slightly higher risk than females. Serologic surveys indicate that subclinical infections are not uncommon, and the case-infection ratio is between 1:25 and 1:200. Antibody prevalence varies widely with region but is in the range of 10 to 30% in adults living in endemic areas of Eastern Europe.

Transmission. RSSE occurs between May and August, whereas CEE cases occur between May and October. The natural transmission cycle involves larval and nymphal ixodid ticks and wild rodents and insectivores (98). *Ixodes ricinus* (the species also responsible for the transmission of

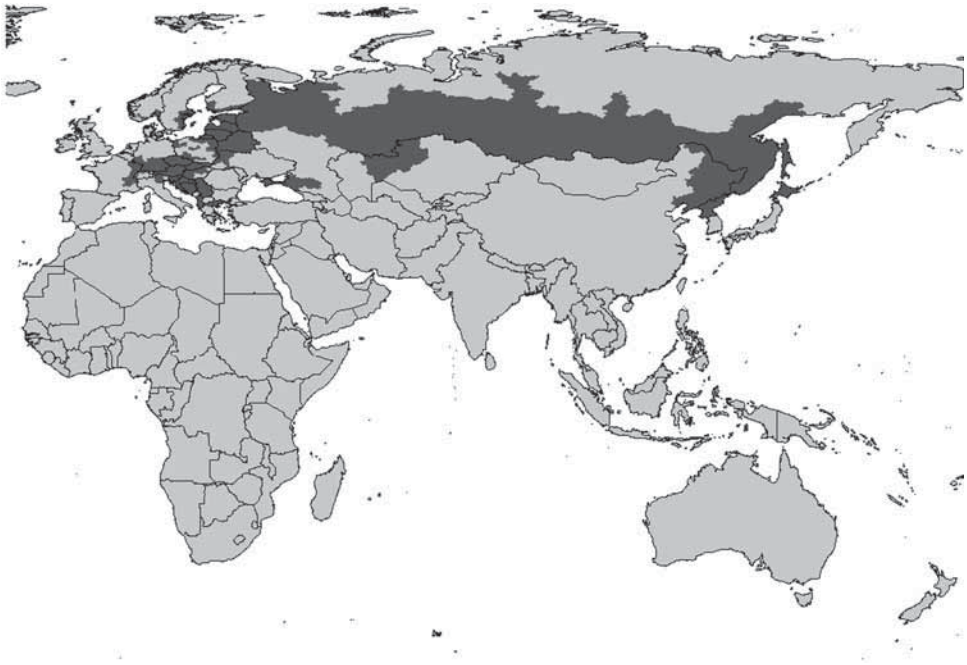

FIGURE 6 Geographical distribution of TBE.

Lyme disease in Europe) and *Ix. persulcatus* are responsible for transmission in Europe and Russia, respectively (Fig. 7). *Ixodes ovatus* is the vector in Hokkaido, Japan. In nature, ixodid vectors may reach high population densities (100–500/m^2), with up to 20% of the ticks infected with virus. Mixed infections of ticks with *Borrelia burgdorferi* and tick-borne encephalitis viruses are common, and concurrent infection of humans with both diseases have been reported.

The active transmission cycle is initiated in the spring when adult and nymphal ticks emerge from hibernation. Adult ticks feed on large mammals, including domestic livestock, and lay eggs that produce larvae, a small proportion of which are transovarially infected. Goats, sheep, cows, and other large mammals are critical to maintaining vector population, but these hosts are not involved, or are rarely involved, in virus transmission to ticks. Infected overwintering nymphal ticks feed together with larval ticks on small rodents and insectivores. Rodents may develop viremia and infect attached ticks, but direct transmission apparently also occurs between infected and uninfected ticks co-feeding on small mammals in the absence of detectable viremia (99). These mechanisms for passage from nymphal to larval ticks ensure amplification of the virus. CEE and RSSE viruses are passed from larva to nymph and from nymph to adult during tick molting. Human infection occurs on exposure to adult ticks that were infected during the previous or current season.

Infected sheep and goats secrete virus, with concentrations as high as 10^5 PFU/ml in milk, and human outbreaks with RSSE or CEE viruses have followed ingestion of unpasteurized sheep's or goat's milk or cheese. Since flaviviruses are inactivated by gastric acid and intestinal bile, it is likely that virus entry occurs in the oropharynx, presumably via tonsillar lymphoid tissue. Laboratory infections with RSSE and CEE viruses occurred frequently, often by the aerosol route, before the use of biological containment procedures and vaccines. Regardless of the route of infection, the patient is not contagious and does not perpetuate transmission by ticks or interhuman contact.

Pathogenesis

Pathological findings are limited to the CNS and are generally similar to those in other flavivirus encephalitides, with a predilection for damage to brainstem structures. Lymphocytic infiltration of the meninges and perivascular inflammation, neuronal degeneration and necrosis, neuronophagia, and glial nodule formation around necrotic neurons are widely scattered throughout cerebral and cerebellar cortices, brain stem, basal ganglia, and spinal cord. The anterior horn cells of the cervical cord are especially affected in RSSE, which explains the lower motor neuron paralysis of the upper extremities, a hallmark of the disease. Indeed, the pathological picture and clinical manifestations (flaccid paralysis) may lead to confusion with poliomyelitis, but, in TBE, the cerebral cortex is more heavily involved. Apoptosis has been proposed as one mechanism of cell death in TBE (100). An interesting feature of members of the TBEV complex is their propensity to cause persistent infections in experimentally infected monkeys, hamsters, and, possibly, humans. Chronic infection in animals is characterized by mutations in the virus (possibly related to evasion of the immune response) and destructive changes to neurons and other cell types in the brain in the absence of significant inflammation. Chronic progressive human encephalitis and seizure disorders in humans (including "Kozhevnikov epilepsy," a form of epilepsia partialis continua) have been associated with RSSE virus (101), and virus has been isolated from the CSF of a patient with a disease clinically resembling amyotrophic lateral sclerosis (102).

Clinical Manifestations

Up to two-thirds of patients have a history of tick-bite. The incubation period is 7 to 14 days. CEE and TBE-Siberia usually have a biphasic course, with an initial grippe-like illness lasting about a week and characterized by fever of 38 to 39° C, chills, malaise, headache, arthralgia, myalgia, and vomiting. This is followed by remission with fatigue but no other symptoms and lasts 1 to 20 days. Most patients

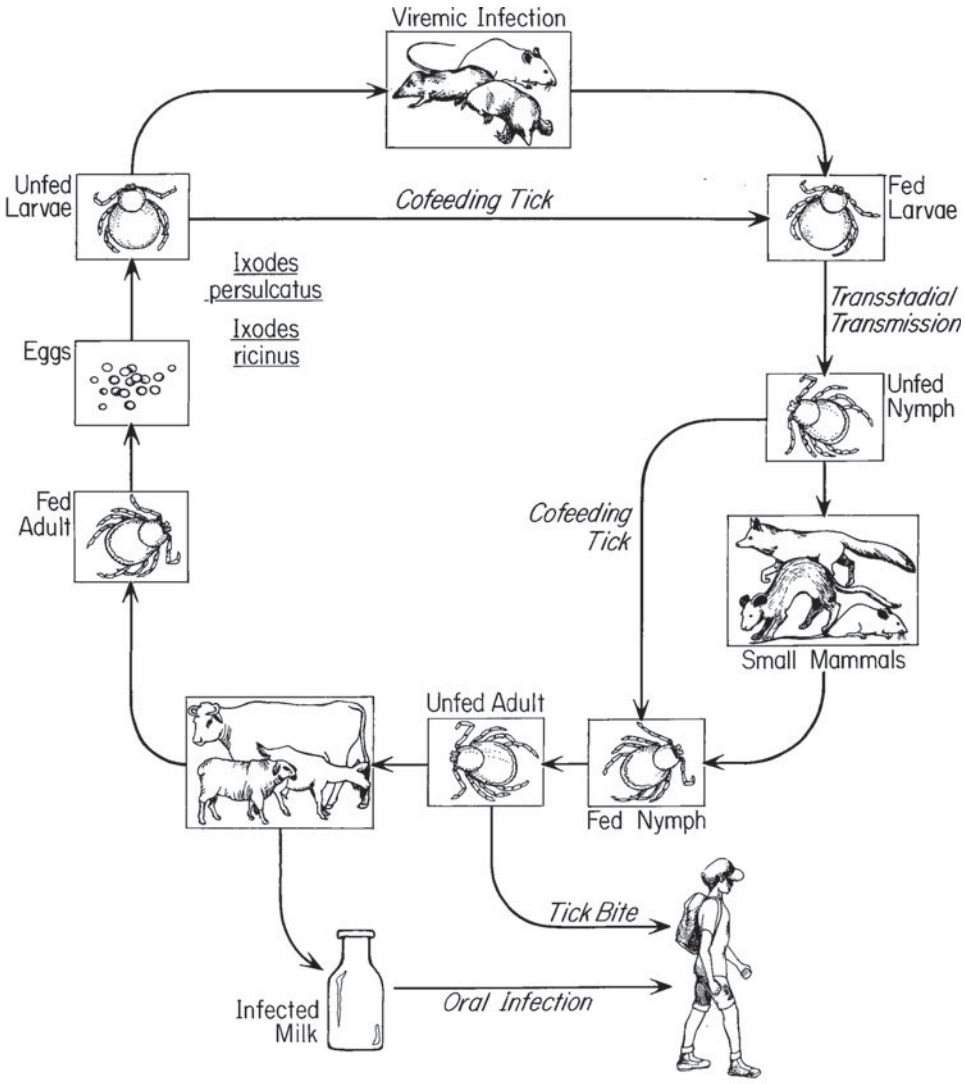

FIGURE 7 Transmission cycle of TBE. Reprinted with permission from T. P. Monath and E. X. Heinz. 1996. Flaviviruses, page 994. In B. N. Fields, D. M. Knipe, and P. M. Howley (eds.), *Fields Virology*, third edition. Lippincott-Raven, Philadelphia, PA.

experience an abortive infection and recover fully without further symptoms. However, up to one-third of patients develop a second phase of the disease, which takes the form of aseptic meningitis in 10 to 50% and encephalitis, myelitis, or radiculitis in the remainder (98). Encephalitic signs include alteration of consciousness, ataxia, paresis or paralysis, and cranial nerve abnormalities. The case-fatality rate is 1 to 3% for CEE and can be as high as 8% for TBE-Siberia. Patients usually die within 1 week of onset of neurological signs. The disease is less severe in children than in adults. Adult patients have higher levels of albumin and specific IgG in the CSF indicating increased virus replication, and they more often require treatment for brain swelling than children. Furthermore adults aged at least 60 years had a more severe initial illness and less favorable outcome than younger adults (103). The CSF changes in TBE are similar to other virus encephalitides. Other clinical laboratory abnormalities include leukocytosis and elevated erythrocyte sedimentation rate. Abnormalities in the EEG and MRI are seen in most patients with encephalitis signs.

About 10 to 30% of patients with acute involvement of brain parenchyma have neuropsychiatric sequelae (98). Postencephalitic signs and symptoms correlate with severity of the acute disease, and patients with coma or a requirement for assisted ventilation have increased sequelae. Objective neurological sequelae (radicular signs and paresis) generally resolve over several months, but inability to work, chronic fatigue, memory and concentration deficits, and other cognitive changes may persist for years. Chronic disease is seen in 1% of TBE-Siberia cases, and these can have evidence of persistent virus infection.

In RSSE the clinical disease is usually monophasic; the onset of illness is more often gradual than acute, with a prodromal phase including fever, headache, anorexia, nausea, vomiting, and photophobia. These symptoms are followed by stiff neck, sensorial changes, visual disturbances, and neurologic dysfunction. The neurologic features in an individual case with encephalitis are highly variable. These include predominantly cerebrocortical forms with obtundation, convulsions, paresis or paralysis, and hyperkinesia; predominant involvement of brain stem with respiratory

depression, cardiac arrhythmia, oculomotor deficits, and paralysis of the shoulder girdle and upper extremities; and myelitic forms with lower motor-neuron paralysis and little alteration of consciousness. Kozhevnikov epilepsy may occur in the acute stage or become part of a chronic postencephalitic syndrome. In fatal cases, death occurs within the first week after onset. Case-fatality rates as high as 20% for RSSE have been reported but may be overestimated by the lack of hospitalization of patients with milder cases. The case fatality rate of the Siberian subtype appears to be similar to that of CEE. Neurologic sequelae, including acute flaccid paralysis of the shoulder girdle and arms, resembling the sequelae of poliomyelitis, occur in as many as 30 to 60% of survivors of RSSE.

Diagnosis

Diagnosis is most often made by serological testing, especially the detection of serum and CSF IgM antibodies by ELISA. A ≥4-fold increase of ELISA antibody titers in paired sera collected two weeks apart is considered a confirmatory diagnosis. IgM antibodies in serum may be detectable for up to 10 months after disease onset. The virus (or viral antigen) may be isolated from the blood during the first phase of illness and from brain tissue of patients dying during the early phase of the disease, but the success rate is less than 10%. RT-PCR on brain tissue may be useful in postmortem diagnosis.

Prevention and Treatment

Vaccination is warranted in persons living in endemic areas, persons working under high-risk conditions, or travelers engaged in high-risk activities. The latter situation prevails in highly endemic countries in eastern and central Europe and is enhanced with occupational or recreational risk factors.

In Europe, two equivalent, safe, and effective inactivated TBE vaccines, based on CEEV strains, are available in adult and pediatric formulations: FSME-IMMUN (Pfizer) and Encepur (GSK). Three inactivated TBE vaccines, based on RSSEV strains, are available: TBE-Moscow (Chumakov Institute of Poliomyelitis and Viral Encephalitides, Moscow, Russia); EnceVir (Microgen, Tomsk, Russia); and a vaccine produced by Changchun Institute of Biological Products, Changchun, China. The TBE-Moscow vaccine is used domestically and internationally, whereas the other two RSSE-based vaccines are used in domestic markets only. Both European vaccines have a two-dose primary series consisting of immunizations administered intramuscularly, starting on day 0, with the second dose given 1 to 3 months later. A booster dose is administered 5 to 12 months (FSME-IMMUN) or 9 to 12 months (Encepur) after the second immunization. For the European vaccines, subsequent booster doses are recommended every 5 years for persons younger than 60 years and every 3 years for persons 60 years or older. For travelers, an accelerated dosing schedule at 0 and 14 days, followed by a booster dose at 5 to 12 months is available. Both Russian vaccines have a two-dose primary series with two doses administered 1 to 7 months apart with a booster 12 months after the second dose and every 3 years thereafter. Vaccine efficacy for both products is estimated at greater than 95%. No tick-borne encephalitis vaccine is licensed in the United States but FSME-IMMUN is licensed in Canada.

The use of repellents or protective clothing may reduce the risk of tick bite, although this is not a practical prophylactic measure for persons with a permanent risk of exposure. Permethrin-impregnated clothing is effective in repelling ticks during outdoor exposure (whereas N, N-diethyl-m-toluamide [DEET] is less effective). Treatment is supportive.

West Nile Virus

WNV was first isolated from the blood of a patient with fever in Uganda in 1937. It is one of the most widely distributed of all arboviruses, but it had not been identified in the New World before its discovery in 1999 in the area of New York, NY (104).

Biology

WNV, a member of the JE antigenic complex, is represented by a single serotype. Sequence analyses suggest that WNV strains can be categorized into at least five phylogenetic lineages (105); however, only lineage 1- and 2-strains have been associated with significant disease outbreaks in humans. The lineage 1 viruses can be further subdivided into three sublineages (a–c): isolates from the Western Hemisphere, Africa, the Middle East, and Europe constitute lineage 1a; Kunjin virus from Australasia represents lineage 1b; and lineage 1c consists of viruses from India (105, 106). The lineage 1a viruses are the most widely dispersed and epidemiologically important, having caused large outbreaks in Europe, Russia, and North America during the past two decades. Lineage 1a can be further subdivided phylogenetically into several clusters, each with a relatively distinct geographic focus of circulation (105). Nevertheless, all but one cluster contains isolates from Africa, suggesting frequent gene flow from Africa to Europe and Russia, most likely by migrating birds.

The means and origin of the lineage 1a WNV strain (East Coast genotype), brought into North America, are unknown. Recent phylogenetic analysis suggests that this virus, and similar strains causing recent outbreaks in Europe, share a common progenitor from North Africa (105). The WNV strain imported into North America contains a single nucleotide change in the NS3 gene (T249P), which confers avian virulence in otherwise nonvirulent strains (107). Since approximately 2002, the East Coast genotype has largely been displaced by a newly evolved genotype (WN02) encompassing 13 nucleotide changes and one conserved amino acid substitution in the E protein, compared to the East Coast genotype, resulting in enhanced dissemination and transmission of the WN02 virus in North American mosquito vectors (108, 109).

Since 2004, highly pathogenic lineage 2 WNVs have caused both human and animal disease in South Africa (110) and Europe (111). Isolates from lineage 2 viruses have histidine at the 249 locus of the NS3 gene; however, isolates that have caused large outbreaks in Greece since 2010 contain a proline substitution at this locus (112, 113). Like lineage 1 viruses, phylogenetic analysis suggests multiple introductions of lineage 2 viruses into Europe from Africa.

The virus is maintained in an enzootic cycle between birds and *Culex* mosquitoes (Fig. 4). Wild birds develop prolonged high levels of viremia and serve as amplifying hosts (114). The major mosquito vector in Africa and the Middle East is *Cx. univittatus*, with *Cx. poicilipes*, *Cx. neavei*, *Cx. decens*, *Aedes albocephalus*, or *Mimomyia* spp. important in some areas. In Europe, *Cx. pipiens*, *Cx. modestus*, and *Coquillettidia richiardii* are important. In Asia, *Cx. tritaeniorhynchus*, *Cx. vishnui*, and *Cx. quinquefasciatus* predominate.

In the United States, more than 65 mosquito species have been infected with WNV. WNV and SLE virus share the same maintenance vectors: *Cx. pipiens* and *Cx. restuans*

in the northeastern United States and Canada, *Cx. quinquefasciatus* in the southern United States, and *Cx. tarsalis* in the western United States and Canada. *Cx. nigripalpus* may be important in Florida. There is evidence from Texas and California that WNV has displaced SLE virus in *Culex* vectors.

Culex mosquitoes are important for their role in overwintering the virus in temperate climates, where they hibernate as adults. The amplification cycle begins when infected overwintering mosquitoes emerge in the spring and infect birds. Amplification within the bird-mosquito-bird cycle continues until late summer and fall, when *Culex* mosquitoes begin diapause and rarely blood feed. The importance of vertical transmission in maintaining the virus is unknown, but it may be an important mechanism of infection of mosquitoes entering diapause.

In the Old World, naturally acquired disease in wild birds is uncommon, suggesting a balanced relationship between virus and hosts. A notable exception was mortality in domestic geese and migrating storks (115) in Israel resulting from infection by a strain similar to the East Coast strain in the United States. WNV has caused lethal infection in more than 320 resident and exotic avian species and marked declines in populations of highly susceptible species, such as American crows, in the United States (116).

WNV grows and produces CPE or plaques in primary chicken embryo cells and in continuous cells of human, primate, swine, and mosquito origin. Mice and hamsters are susceptible to lethal infection by the intracerebral and intraperitoneal routes. Equine encephalitis outbreaks have occurred in many countries. In the United States, equine incidence peaked in 2002 and subsequently decreased after equine WNV vaccines became available. Approximately 10% of experimentally infected horses develop clinical illness. Ill horses experience a 20 to 40% mortality rate. Age, vaccination status, inability to rise, and female gender are associated with the risk of death (117). Viremia in horses is low and of short duration; thus, horses are unlikely to serve as important amplifying hosts for WNV in nature.

Epidemiology

Distribution and geography. WNV is present throughout Africa, the Middle East, continental Europe, India, Indonesia, and Australia. Notably, the virus has rarely been identified from JE endemic areas. In the New World, its distribution has expanded from its discovery site in New York City in 1999 to Argentina in 2006. Despite serological evidence of WNV circulation in many countries in the Caribbean and Latin America, human and equine disease has rarely been reported.

Before 1995, human infection with WNV was mainly associated with little or no disease. In the Nile Delta of Egypt, serological data showing 6% seroprevalence in schoolchildren and 40% in young adults suggest that infection is very common (118). In 1974, Cape Province, South Africa experienced an outbreak with a 55% incidence of infection with thousands of clinical cases, yet illness was mild and without encephalitis. Outside of Africa, small outbreaks or sporadic cases of CNS infection had been reported from Israel, India, and several European countries (119). A marked upswing in WNV activity began in the mid-1990s coincident with the emergence of a group of genetically similar lineage-1 viruses of apparent increased pathogenicity (120, 121). Outbreaks associated with a high incidence of severe neuroinvasive disease occurred in Algeria (1994), Romania (1996), Tunisia (1997), Russia (1999 to 2001), the United States (1999), Israel (2000), and Sudan (2002) (122). Subsequently, outbreaks of CNS infection from both lineage 1 and 2 viruses have continued in parts of Europe.

Incidence and Prevalence. In the New World, significant human morbidity and mortality have been noted only in the United States and Canada. The initial outbreak in New York City caused 62 cases and 7 deaths in Queens and surrounding areas (104). The virus then spread rapidly from its initial locus (Fig. 8). Surveillance systems focus on detection of WNV neuroinvasive disease (encephalitis, meningitis, acute flaccid paralysis) since reporting of these cases is relatively complete. From 1999 to 2014, a total of 41,762 confirmed and probable cases of WNV disease, including 18,810 cases of neuroinvasive disease, were reported to the CDC from 48 states and the District of Colombia. Only 19 human cases of WNV neuroinvasive disease were reported in the United States in 2000 and 64 in 2001. However, from 2002 to 2014, the number of reported neuroinvasive disease cases ranged from 386 to 2,946, with the three largest outbreaks occurring in 2002 (2,946 cases), 2003 (2,866 cases), and 2012 (2,873 cases) (123, 124). Neuroinvasive disease varies considerably across the United States, with the highest-incidence states located in the Midwest. In Canada, 1,030 patients with neuroinvasive disease were reported through 2014.

Serologic surveys and extrapolations from blood-donor screening data indicate that neuroinvasive disease following WNV infection is infrequent, with estimates ranging from 1 in 140 to 1 in 256 infections resulting in meningitis or encephalitis (125–127). By extrapolation, the 18,810 cases of neuroinvasive disease reported in the United States through

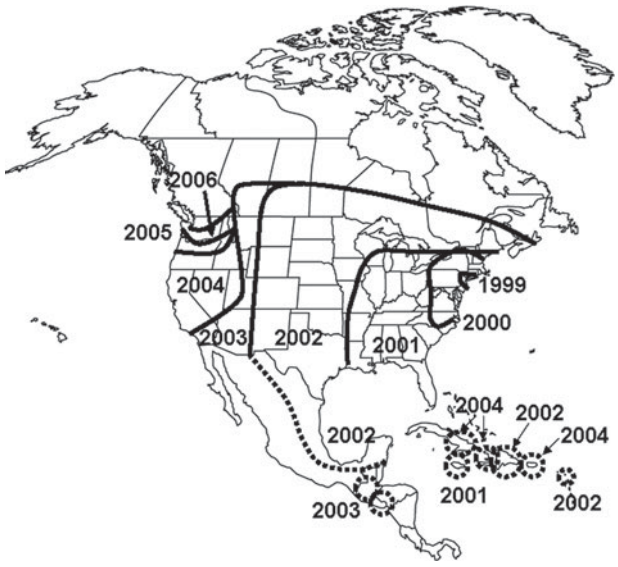

FIGURE 8 Expansion of WNV in North America, Central America, and the Caribbean, 1999 to 2006. Areas demarcated by solid lines confirmed by virological confirmation in mosquitoes or vertebrates. Areas demarcated by dashed lines are those with serological evidence in vertebrates only. Serological evidence of WNV was found in Colombia and Venezuela in 2004, and WNV caused an equine outbreak in Argentina in 2006.

2014 would imply that from 2.6 to 4.8 million persons have been infected. Serological surveys indicate that even in areas experiencing outbreaks, less than 10% of the population has been exposed to the virus (126–129).

Human WNV infection incidence increases in early summer and peaks in August or early September. In the United States, within large regional WNV epidemics, the incidence of human disease varies markedly from county to county, suggesting the importance of local ecological conditions. Urban and agricultural land covers, rural irrigated landscapes, farming activities, and several socioeconomic factors relate to higher WNV incidence in some locations. Males and persons living in a rural area were approximately 1.4 times and 3.4 times (respectively) more likely than females or persons living in suburban or urban locations to have WNV infection (130).

Risk Factors Associated with Human Disease. Serologic surveys in Romania (128) and New York City (127, 128), as well as blood-donor screening data (131), indicate that WNV infection incidence is constant across all age groups during outbreaks. Advancing age profoundly increases the risk of neuroinvasive disease, particularly encephalitis (126, 132). The risk may approach one in 50 among persons aged at least 65 years, a rate 16 times higher than that for persons aged 16 to 24 years (126). In addition, a history of cancer, diabetes, hypertension, alcohol abuse, renal disease, and *CCR5* deficiency, as well as male sex, may increase the risk of neuroinvasive disease (126, 132–136). Persons infected through transplantation of WNV-infected organs are at extreme risk of developing neuroinvasive disease (137), but conflicting data exist regarding risk among previous organ recipients infected via a mosquito bite (138, 139).

Transmission

Mosquito bites account for nearly all human WNV infections. West Nile virus can also be transmitted via transfused platelets, red blood cells, and fresh frozen plasma (140), as well as through heart, liver, lung, and kidney transplantation (137). Transmission via organ transplantation has occurred from donors without detectable viremia, suggesting viral sequestration in organs shortly after viremia has cleared.

One possible transplacental transmission following a second trimester WNV infection resulted in an infant with chorioretinitis, lissencephaly, and cerebral white matter loss (141). Fortunately, fetal abnormalities due to intrauterine infection are uncommon; none of 72 live infants born to 71 women infected during pregnancy had malformations linked to WNV infection or had conclusive laboratory evidence of congenital WNV infection (142). Nevertheless, three infants born to women infected within three weeks prepartum developed symptomatic WNV disease at, or shortly after, birth, indicating the possibility of intrauterine infection or infection at the time of delivery. Other rare or suspected modes of transmission include breast milk, percutaneous or conjunctival exposure to laboratory workers, and by unknown means in dialysis-unit patients and workers at a turkey breeder farm (143).

Pathogenesis

Mosquito salivary components introduced at the site of infection in vertebrates modulate initial infection of target cells like keratinocytes (144) and skin-resident dendritic cells through several mechanisms, including focal suppression of immune effector cell trafficking to the site of inoculation (145). Infected dendritic cells or keratinocytes migrate to draining lymph nodes from which a serum viremia is generated that then relays infection to visceral organs and potentially to the central nervous system (CNS).

WNV is capable of replicating and eliciting pathology in the brain, but a critical prerequisite to generating neuroinvasive disease in humans is the virus' capacity to gain access to the CNS (i.e., neuroinvasiveness). Postulated WNV neuroinvasive mechanisms include direct viral crossing of the blood-brain barrier (BBB) due to cytokine-mediated increased vascular permeability, a Trojan horse mechanism in which infected tissue macrophages are trafficked across the BBB, direct infection and passage through the endothelium of the BBB, and retrograde axonal transport of the virus to the CNS via infection of olfactory or peripheral neurons (136). Regardless of how WNV enters the CNS, murine models of infection have shown persistent viral replication in various tissues, including the CNS, suggesting a potential etiology for long-term neurological sequelae observed in neuroinvasive-disease patients (136).

WNV could be isolated from lung, heart, skeletal muscle, skin, lymph nodes, liver, small intestine, kidney, thyroid, spinal cord, pons, basal ganglia, cerebellum, and cerebrum in patients who died within one month of receiving WNV for treatment of cancer during the 1950s (146). In one study, all 23 patients who died from WNV encephalomyelitis had glial nodules with variable loss of neurons and perivascular cuffing by mononuclear cells (147). Mononuclear inflammation and loss of neurons were most prominent in the gray matter of the medulla, pons, and midbrain. Inflammation of spinal cord was universally present, particularly in the anterior horns. Glial nodules and lymphocytic perivascular cuffing were usually seen in both anterior and posterior horns. Immunohistochemical assays showed viral antigens in the cytoplasm of neurons and neuronal process.

Clinical Manifestations

The typical incubation period for infection ranges from 2 to 14 days, although longer incubation periods have been observed among immunosuppressed hosts (140). Most persons infected with WNV are asymptomatic, because symptoms are seen in only about 25 percent of infected patients (148).

West Nile Fever. The usual presentation is a self-limited febrile illness, called West Nile fever, which is indistinguishable from dengue and other viral fevers. The illness is characterized by fever, headache, malaise, back pain, myalgias, and anorexia persisting for three to six days. Eye pain, pharyngitis, nausea, vomiting, diarrhea, and abdominal pain can also occur. A rash, which often appears around the time of defervescence, tends to be morbilliform and nonpruritic, predominating over the torso and extremities, but sparing the palms and soles.

Although initially considered a mild, febrile illness, many patients experience a more severe and prolonged course. In a study of 98 patients with West Nile fever, the frequency and median duration of symptoms were as follows: fatigue (96%; 36 days), fever (81%; 5 days), headache (71%; 10 days), muscle weakness (61%; 28 days), and difficulty concentrating (53%; 14 days) (149). Thirty patients required hospitalization for a median of five days, and 79% missed work or school for a median of 10 days. At 30 days, 63% were symptomatic. In a follow-up study of 531 patients with West Nile fever, 53% reported symptoms of at least 30 days in duration, and 79% reported missing work for a median of 16 days (150).

Neuroinvasive Disease. Approximately one in 150 to 250 persons infected with WNV develop neuroinvasive disease (127, 151), which is manifested by meningitis, encephalitis, or acute flaccid paralysis. Encephalitis that is associated with muscle weakness and flaccid paralysis is particularly suggestive of WNV infection. West Nile encephalitis ranges in severity from a mild, self-limited confusional state to severe encephalopathy, coma, and death. Fever is present in at least 90%, with weakness, nausea, vomiting, and headache in approximately one-half of patients. Other neurologic manifestations include tremor, myoclonus, and parkinsonian features, such as rigidity, postural instability, and bradykinesia. The case-fatality rate among persons with neuroinvasive disease is approximately 10%.

Although Guillain-Barré syndrome can occur, WNV-associated acute flaccid paralysis most commonly results from destruction of anterior horn cells. Paralysis from WNV myelitis is asymmetric and can occur without overt meningitis or encephalitis (152). Cranial nerve palsies can occur, resulting in facial weakness, vertigo, dysarthria, and dysphagia. Facial paralysis carries a favorable prognosis. Dysarthria and dysphagia accompanied by acute flaccid paralysis indicate a high risk of impending respiratory failure (152). All patients have some improvement in muscle strength, mostly in the first 4 months following acute illness, with about one-third having significant recovery and one-third recovering fully. Patients with respiratory failure experience > 50% mortality, although functional recovery is highly variable and complete recovery is possible (153). WNV infection infrequently causes other forms of weakness, including brachial plexopathy, radiculopathy, and a predominantly demyelinating peripheral neuropathy similar to Guillain-Barré syndrome. Other neurologic complications with WNV include seizures, cerebellar ataxia, optic neuritis, and hearing loss.

Total leukocyte counts in peripheral blood are mostly normal or elevated. In cases with signs of CNS involvement, the CSF usually demonstrates a pleocytosis often with a predominance of lymphocytes, as well as an elevated protein concentration. A few patients with meningitis or encephalitis have normal (< 5 per mm^3) CSF cell counts (3 and 5%, respectively). Over 90% of patients had elevated CSF protein levels.

CT of the brain typically shows no evidence of acute disease. MRI shows abnormalities in 20 to 70% of patients (154). Hyperintensity on T2-weighed MR images may be seen in regions such as the basal ganglia, thalami, caudate nuclei, brainstem, and spinal cord. Electroencephalography (EEG) in patients with meningitis or encephalitis typically shows generalized, continuous slowing, which is more prominent in the frontal or temporal regions (155). Patients with acute flaccid paralysis have electrodiagnostic studies showing normal sensory nerve action potentials (SNAPs) with compound motor action potentials (CMAPs) varying between normal and markedly decreased, depending on the degree of paralysis (156).

Other Complications. Other manifestations described with WNV infection include multifocal choroiditis, vitritis, myocarditis, pancreatitis, fulminant hepatitis, rhabdomyolysis, stiff-person syndrome, and autonomic instability (143).

Diagnosis

WNV should be considered in patients who have unexplained febrile illness, encephalitis, meningitis, or acute flaccid paralysis during mosquito season. Evidence of WNV enzootic activity or other human cases, either locally or in a region where the patient has traveled, should raise the index of suspicion.

Viremic blood donors develop IgM antibody in plasma within nine days after donation (157). Serum or CSF can be tested with the IgM antibody capture ELISA. Nearly all patients with meningitis or encephalitis have a positive IgM antibody capture ELISA result at clinical presentation, but patients with West Nile fever may not have demonstrable antibody in serum obtained within eight days of clinical onset and can only be documented to have West Nile virus infection after nucleic acid amplification test or convalescent-phase samples demonstrate antibody (158). IgM antibody to WNV may persist for 1 year or longer. False positive ELISA results can occur due to recent immunization with yellow fever or JE vaccines or due to infections with other related flaviviruses, such as SLE or dengue viruses.

WNV can be isolated or viral antigen or nucleic acid detected in CSF, tissue, or other body fluids, although the low sensitivities of these methods preclude their use as routine screening tests. Nucleic acid testing (NAT), such as real-time PCR, has been positive in up to 55% of CSF samples. NAT of plasma from blood donors indicates that viremia is of a low titer and detectable for a median of 6.9 days (159). Among patients with neuroinvasive disease, NAT of plasma has a sensitivity of less than 15% (104, 158). However, patients with West Nile fever have been diagnosed by NAT, serology, or a combined approach of these two methods in 45, 58, and 94% of cases, respectively (158). Thus NAT may complement serologic testing in patients with suspected West Nile fever, particularly if the test is available and urgent diagnosis is required. NAT in serum or CSF may be valuable for severely immunocompromised patients, who may have prolonged viremia or lack IgM antibody.

Treatment

Treatment of WNV infection remains supportive. Several investigational therapeutic approaches including immune γ-globulin, WNV-specific neutralizing monoclonal antibodies, corticosteroids, ribavirin, interferon α-2b, and anti-sense oligomers (160, 161) have failed to document efficacy, in part due to difficulty in recruiting sufficient numbers of patients. Case reports or uncontrolled clinical series suggesting efficacy should be interpreted with extreme caution due to WNV's highly variable clinical course. There is intensive research using structure-function studies of virus-encoded proteins to identify viral targets for drug development, but these are all *in vitro* or small animal models studies. (162–165), including Favipiravir (T-705), which is a broad spectrum RNA virus inhibitor (166).

Prevention

The introduction of a vaccine against WNV for use in horses has substantially reduced the incidence of equine WNV disease in the United States (167, 168). Three licensed inactivated vaccines are available in the United States or the European Union (169–173); a DNA vaccine has also been licensed in the United States but is not commercially available (174).

Several human WNV vaccine constructs have been developed, some of which have reached human clinical trials (173). Chimeric vaccines, inserting WNV prM and E protein genes into attenuated yellow fever and dengue 4 virus backbones, have undergone successful phase 2 and 1 clinical

trials, respectively, and have demonstrated safety and immunogenicity, although efficacy trials were not possible (175). A phase 1 WNV DNA vaccine trial has been completed demonstrating safety and immunogenicity (176). Phase-3 efficacy trials have not been attempted due to the unknown market potential of a WNV vaccine and logistical difficulties in conducting phase-3 clinical trials for this sporadic and widely dispersed disease (160).

Personal protection to avoid mosquito exposure and the use of repellents is advised during periods of WNV activity. Public health measures include surveillance for virus activity in birds, mosquitoes, and humans, and the use of pesticides to kill adult infected mosquitoes.

Flaviviruses Associated Primarily with Febrile Syndromes with Rash

Dengue Viruses

Dengue disease was first described in the 18th century, but the causative agent was not isolated until 1944. Between 1944 and 1956 it was shown that four distinct viruses, designated dengue types 1 to 4, were responsible for the same clinical syndrome. In 1956, a severe form of the disease, dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), was described for the first time, although, in retrospect, earlier isolated outbreaks had probably occurred in Australia and South Africa. Both dengue fever and DHF/DSS are increasing in frequency worldwide.

Biology

Dengue fever is caused by four distinct antigenically and genetically related viruses (dengue virus types 1 to 4) that are distinguished by neutralization tests. Infection with an individual dengue serotype confers lifelong protection to that serotype, but sequential infections with different serotypes occur because cross-protection between serotypes *in vivo* is absent or of short duration. Sequences of strains of dengue virus belonging to the same serotype are >90% homologous, whereas homology across serotypes is approximately 65% (177). Distinct genotypes have evolved in different geographic regions, and genotyping thus provides a means of determining the origin and spread of epidemics (178). In many areas of the world, two or more genotypes of each serotype co-circulate, reflecting either the evolution of autochthonous variants over time, or introduction and spread of a distinct genotype from another region. In addition to mutational events, intertypic recombination appears to play a role in the evolution of variants within dengue serotypes (4) but not between serotypes.

All four dengue virus serotypes cause DHF/DSS, but dengue type 2 is the most important, followed by dengue type 3 (179–181). The sequence of infecting serotypes and strain differences in virulence appear to influence the occurrence of DHF. There is no animal model of DHF disease permitting study of these factors. Epidemiological studies and comparison of nucleotide sequences of strains associated with different disease outcomes have provided clues that virus specified factors may contribute to the pathogenesis of DHF. In the Americas, two distinct genotypes of dengue 2 virus have circulated since 1981, when a strain belonging to the genotype I was introduced from Southeast Asia and superimposed on the endemic "American" genotype V (179). The Asian genotype has caused multiple epidemics of DHF/DSS in populations with prior immunity to dengue 1 (182), whereas the American genotype appears to be less pathogenic and has not caused outbreaks of severe disease in populations with a similar immunological background (183). A detailed comparison of the nucleotide sequences of multiple dengue 2 strains, representing Southeast Asian genotype I and American genotype V, led to the identification of structural differences at critical determinants that were associated with the potential to cause DHF. These differences were located at E390 (putative cell receptor binding region of the E protein, a region of the 5' noncoding region (NCR) involved in the initiation of translation, and a region of the 3'NCR believed important in the formation of replicative intermediates (184).

The structure and replication strategy of dengue viruses are similar to other flaviviruses, but their host range and growth characteristics *in vitro* are distinctive. Unadapted isolates of dengue virus often do not cause CPE in cell culture and are not pathogenic for infant mice. Neuroadaptation by sequential passage is required to produce consistent pathogenicity. In general, cells of monkey (Vero, LLC-MK2) and mosquito (*A. albopictus* C6/36) origin are most useful for virus isolation and propagation *in vitro* (Table 3). Syncytium formation occurs in mosquito cells. Dengue viruses replicate to high titer in mosquitoes after intrathoracic inoculation.

Epidemiology

Distribution and Incidence. Dengue viruses occur worldwide in tropical regions. Their distribution is determined by the presence of the principal mosquito vector (*Aedes aegypti*) (Fig. 9). Since World War II, dengue has expanded in incidence and geography, due principally to urbanization and the attendant increase in domestic *A. aegypti* populations, and to the increased movement of infected people by airplanes (185). It is estimated that over 3.9 billion people in 128 countries inhabit tropical areas at risk of infection (186) and about 390 million dengue virus infections occur annually throughout the world, resulting in 96 million cases of dengue disease (187).

Dengue infections are most prevalent in Southeast Asia, where all four serotypes are continuously present. In recent years, the Indian subcontinent, southern China, and Taiwan have experienced epidemics. Island nations in Oceania have long been at risk of epidemic spread. All four serotypes have been introduced episodically, but the island outbreaks have generally been caused by a single serotype. Intermittent transmission of dengue has occurred in Queensland, Australia. All four serotypes have been isolated in tropical Africa, but their medical impact remains unclear due to poor surveillance.

Many areas of Central and South America, the Caribbean, and the coastal United States experienced outbreaks throughout the 19th and early 20th centuries. Concerted control programs in the mid-20th century effectively eliminated the *A. aegypti* mosquito from much of the Americas, and dengue activity was reduced to sporadic activity in the Caribbean (188). However, discontinuation of the control program in the 1970s resulted in reinfestation of *A. aegypti* to its former range by the mid-1990s. This has resulted in an increase in annual reported dengue cases from 66,000 in 1980 to more than a million cases in recent years over a widening geographic range (189).

Dengue epidemics have occurred occasionally in the continental United States since the end of World War II (Louisiana in 1945; Texas in 1980, 1986, 1995, 2000, and 2005; Florida 2009, 2010, 2013). The Texas outbreaks resulted from amplified transmission in northern Mexico and

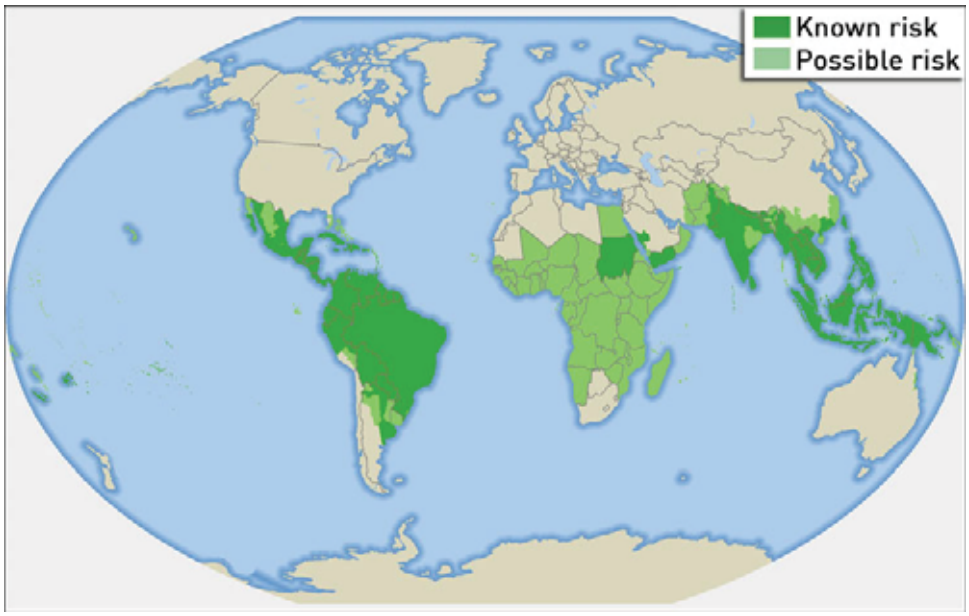

FIGURE 9 Geographical risk map of dengue fever.

included both imported cases and cases derived from secondary spread (190). Following a 56-year hiatus, an outbreak vectored by the A. *albopictus* mosquito occurred in Hawaii from 2001 to 2002 (191).

In hyperendemic areas of Southeast Asia, the annual incidence of infection is 10 to 20%, and most children have experienced at least one dengue infection by the age of 7 years. In these areas, dengue is a childhood disease, and adults are protected by cumulative immunity. In island communities or in naïve populations undergoing virgin-soil epidemics, the incidence of dengue infection has been 70% or higher, with similar attack rates across all age groups. The ratio of inapparent/apparent infections is probably highly variable due to age, other host factors, and virus strain variation. Inapparent-to-apparent infection ratios are between 6:1 and 15:1 for primary infection (187, 192) and 1:1 to 3:1 in adults (193–195). In a dengue-naïve population, the risk of dengue fever following primary infection increases with age (196).

The WHO estimates that 500,000 people with severe dengue require hospitalization annually, with a 2.5% overall mortality. Severe dengue now occurs in many countries every year, with a pattern of increasing incidence, largely as the result of the global transport of dengue viruses resulting in co-circulation of multiple dengue serotypes, permitting secondary infections. Immunity acquired after infection with one serotype confers full (probably lifelong) protection against reinfection with that serotype, but predisposes an individual to more severe disease (DHF) on sequential infection with another dengue serotype. The ratio of inapparent-to-apparent infection is significantly lower in secondary infection.

In the American region, sporadic cases of DHF were reported in the 1970s, and the first epidemic occurred in Cuba in 1981 (10,000 cases, 158 deaths) with the introduction of a dengue 2 Asian genotype virus (182). Subsequently, major outbreaks of severe dengue have been recorded throughout the region as all four dengue genotypes now circulate throughout the Americas.

Transmission. *Aedes* spp. mosquitoes transmit dengue virus, and humans are the intermediate host. A. *aegypti* is the most important vector globally. In tropical areas, vectors are active year round and dengue occurs throughout the year, with increased transmission during the rainy season. This is due to higher mean temperatures and the attendant shorter extrinsic incubation period in the vector and to higher humidity and enhanced survival of adult mosquitoes. In temperate zones, transmission is limited to the summer months. Dengue fever is an important travel-associated infection, and hundreds of imported cases of dengue fever are reported each year in travelers returning to the United States, Europe, and Australia.

The period of viremia for blood-feeding adult female vectors, during which humans are infectious, is 3 to 5 days (197). After blood feeding, an extrinsic incubation period of 10 to 14 days must elapse before A. *aegypti* can transmit the virus upon refeeding (197). A. *aegypti* is a domestic species that breeds in artificial containers and bites in and around human habitation. Peak biting activity occurs in the early morning and late afternoon. In tropical areas where storage of drinking water is practiced, 10 to 20 female A. *aegypti* may be found per room, of which 5 to 10% may be infected with dengue virus. However, outbreaks with vector densities of less than 1 female mosquito per house have also been recorded. A. *aegypti* is a furtive mosquito and may probe several persons before completing a blood-meal, enhancing the rate of virus transmission due to the presence of virus in mosquito saliva. The vector population threshold for dengue transmission has not been established, although it is clear from experiences in Cuba and Singapore that very low vector densities must be reached for transmission to cease (see "Prevention" below). In rural areas in some parts of the world, A. *albopictus* plays a secondary role in interhuman transmission of dengue. A. *albopictus* spread from Asia to the New World in the 1980s, and, in 1995, was implicated in dengue transmission for the first time in Mexico (198).

The maintenance of dengue viruses between epidemics has not been clearly defined, especially in rural areas with

relatively sparse human populations. Vertical transmission of dengue virus in *A. aegypti* and in sylvatic *Aedes spp.* occurs, and probably contributes, to virus maintenance. In Southeast Asia and West Africa, a sylvatic cycle of transmission occurs, involving nonhuman primates and tree-hole-breeding *Aedes* spp., similar to the cycle for yellow fever (Fig. 10). However, in Africa, virus strains involved in the sylvatic cycle are distinct from strains associated with human disease outbreaks (199).

Dengue infections have been transmitted by needle stick, from bone marrow transplantation, and blood transfusion.

Risk Factors Associated with Severe Dengue

The principal risk factor for DHF/DSS is prior exposure to a heterologous dengue serotype. Approximately 90% of persons with DHF/DSS are secondary infections (i.e., a second dengue infection involving a serotype different from that causing the first infection). The risk of DHF is estimated to be 15 times higher and that of DSS 50–100 times higher in secondary than in primary dengue infection (180).

Age influences disease expression. Unlike classical dengue fever, DHF/DSS is principally a disease of childhood. Two peaks have been noted in age-specific incidence rates: children younger than 1 year old and children 3 to 5 years of age. The disease in infants is rare and is associated with primary infection in the presence of maternal antibody, whereas the vast majority of cases in older children are the result of secondary infections. In Thailand the highest incidence of DHF/DSS is in children 5 to 9 years of age. In the 1981 DHF epidemic in Cuba, children had a higher incidence of severe disease compared to adults with a similar immunologic predisposition (200). However, when dengue 2 reinvaded in 1997, all individuals with prior exposure to dengue 1 had been infected at least 9 years before (dengue 1 struck Cuba in 1977 to 1978) and all patients hospitalized were >15 years old (201). The case-fatality rate was higher in this outbreak than in 1981 (182). These findings illustrate the importance of age in DHF pathogenesis.

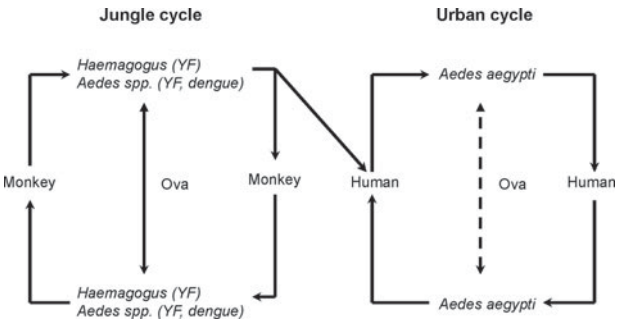

FIGURE 10 Simplified transmission cycle of dengue and yellow fever viruses. Both viruses have a jungle cycle involving tree-hole mosquitoes. In the Americas, *Haemagogus* sp. transmits yellow fever, but no jungle cycle for dengue has been discovered. In Africa, both viruses are transmitted in jungle cycles involving *Aedes* spp. Yellow fever does not exist in Asia, but in Malaysia, Sri Lanka, and probably elsewhere, dengue virus is transmitted in a jungle cycle involving *Aedes* spp. The relationship between the jungle cycle of dengue and human infections is not clear. However, the jungle cycle of yellow fever is a source of human infections in the Americas and Africa. Dengue transmission occurs principally in urban environments, where domestic *Ae. aegypti* serves as the vector and humans serve as the viremic host. A similar cycle of yellow fever transmission occurs commonly in West Africa. No urban outbreaks of yellow fever have occurred in the Americas since 1964, except possibly for a very limited outbreak in Bolivia in 1997 to 1998.

In addition, age, sex, race, and acquired host factors modulate disease expression. The incidence of DHF (but not of dengue infection) is higher in females than in males. It is hypothesized that females may have a more robust immune response to dengue infection or may be more susceptible to cytokine-mediated vascular injury. Malnutrition, which may reduce immune responsiveness, appears to spare children from DHF/DSS. Race influences disease severity; in Cuba, Caucasians and Asians had a significantly higher incidence of DHF/DSS than did blacks (200). Underlying disease or special condition may exacerbate illness. Peptic ulcer disease (202) and menstruation were risk factors for gastrointestinal hemorrhage and menorrhagia, respectively. Underlying diseases (e.g., sickle cell disease, diabetes mellitus, and bronchial asthma) (203, 204) as well as HLA-related and nonrelated host genetics (e.g., Fcγ receptor IIA, G6PD deficiency, TNFα, interleukin 10) (205, 206) increase the risk of developing severe disease.

Pathogenesis

The primary cells infected after inoculation in mosquito saliva are probably dendritic cells in the skin (207), which subsequently migrate to draining lymph nodes (24). After initial replication in skin and draining lymph nodes, virus appears in blood during the acute febrile phase, generally for 3 to 5 days, and may be recovered from serum and from peripheral blood mononuclear cells (208). Although blood-derived dendritic cells are highly permissive to infection, one study indicated the principal subset of PBMCs infected were CD20+ B cells (209). Dengue virus enters cells via clathrin-dependent receptor-mediated endocytosis, with DC-SIGN serving as a virus attachment factor on dendritic cells.

Both the innate and adaptive immune responses are important for controlling dengue virus infection (210). As with other flaviviral infections, toll-like receptors and intracellular sensors, such as the helicases, melanoma differentiation-associated protein 5 (MDA5), and retinoic acid-inducible gene 1(RIG-1) are important in recognizing viral RNA, which, in turn, induces strong IFN responses (211). Dengue viruses have developed mechanisms to downregulate the IFN response, which is a powerful inhibitor to dengue virus replication. After primary infection, a strong virion-specific neutralizing antibody response is directed toward epitopes located on domain II of the viral E protein. However, most antibodies are dengue-serotype cross-reactive but have low or no neutralizing activity, with a few high-titer serotype-specific antibodies.

The viremic period is terminated coincident with the appearance of serum antibodies. In most patients, humoral and cellular responses result in recovery from infection and long-lasting protection against reinfection with the homologous serotype. Serotype-specific neutralizing antibodies directed against the E protein and serotype-specific CD4+ and CD8+ cytotoxic lymphocytes directed against structural and nonstructural virus targets on infected cells are responsible for protection and recovery. Cross-protection against other dengue serotypes is short-lived, and severe disease (DHF/DSS) occurs in a subset of individuals experiencing secondary infection with a heterologous serotype.

The genesis of systemic symptoms in dengue fever is uncertain, but the release of cytokines and chemokines, as a result of virus injury to dendritic cells and macrophages and activation of CD4+ and CD8+ T lymphocytes, play an

important role. In uncomplicated (classical) dengue fever, elevated plasma or blood levels, soluble IL-2 receptor, IL-2, soluble CD4, and interferon-γ, and other cytokines like TNF-α, IL-1β, and platelet activating factor in plasma or blood are present. High levels of interferon-α have been documented for a week or more after the onset of illness. Leukopenia, due to transient bone marrow suppression, is a feature of dengue fever and also may be due to the release of soluble factors from infected monocytes. A 43-kDa cytokine produced by T cells and cytotoxic for mouse splenocytes has been demonstrated in mice and humans and may play a role in the abnormalities of hematopoiesis seen in dengue.

DHF/DSS. Several factors may predispose individuals to the development of DHF/DSS, including primary versus secondary infection, time interval between infections, host genetics, and viral strain. DHF/DSS is characterized by diffuse capillary leakage and hemorrhage. Increased vascular permeability results in hemoconcentration, decreased effective blood volume, tissue hypoxia, lactic acidosis, and shock. These perturbations in homeostasis are mediated by cytokines, and there are consequently few pathologic findings in fatal human cases. On autopsy, signs of capillary leak (pleural effusions, retroperitoneal edema) and of hemorrhage (petechiae, ecchymoses, and visceral hemorrhages) are evident. Histopathologic examination reveals perivascular edema and hemorrhage, proliferation of lymphoid cells and plasmacytoid elements in spleen and lymph nodes, and necrosis of thymus-dependent areas of the spleen. Central or paracentral focal necrosis of hepatocytes, Councilman bodies, hypertrophy of Kupffer cells, and focal mononuclear cell infiltration may be evident in the liver. Bone marrow changes include maturational arrest of megakaryocytes. Focal dengue virus antigen has been demonstrated in skin, liver, and mononuclear leukocytes.

The precise mechanism of DHF/DSS pathogenesis is not well understood, in part due to the lack of a suitable animal model. Proposed mechanisms include (i) antibody-mediated enhancement of infection of monocyte/macrophages; (ii) activation of dengue-specific lymphocytes, with release of cytokines and activation of complement; and (iii) innate immune response, with immune clearance of infected monocyte/macrophages and release of cytokines and activation of complement.

In the setting of a secondary dengue infection, preexisting nonneutralizing antibodies recognizing antigenic determinants on the E glycoprotein that are shared by the four dengue viruses (sometimes termed "heterologous antibody") bind dengue virus and form infectious immune complexes. Dengue virus bound to IgG antibody gains access to the principal cell targets for replication (monocyte/macrophages) via Fc-γ receptors, in particular FcRII. The number of infected cells in the host and the overall virus load is thus enhanced. This phenomenon of antibody-mediated enhancement of virus replication has been demonstrated *in vitro* and in animal models but has been difficult to demonstrate in humans. In addition to a role for the E protein, the NS1 protein may also play a role. NS1 is found in both cell-associated and secreted forms, with the latter associated with viremia in secondary infections. However, studies to date show no correlation between anti-NS1 antibodies and plasma leakage.

Primary infection also induces both serotype-specific and dengue virus cross-reactive $CD4^+$ and $CD8^+$ CTLs directed against cross-reactive structural and (especially) nonstructural virion proteins expressed on the surface of infected monocytes. Activation of dengue-specific T lymphocytes, interacting with infected monocyte/macrophages, causes release of IFNγ, IL-2, TNF-α, and TNF-β. Interestingly, $CD4^+$ T cells produce greater quantities of IFNγ to the homologous virus causing the primary infection, while the ratio of TNF-α to IFNγ is higher following stimulation with heterologous dengue viruses. In terms of secondary infections, it is hypothesized that low-avidity dengue-virus cross-reactive T cells, induced by the primary infecting dengue virus, dominate during a secondary heterologous dengue virus infection. Furthermore, since plasma leakage is relatively short-lived, it suggests a role for the innate immune response, in particular pro-inflammatory mediators, such as IL-6, IL-8, and TNF-α.

The overall numbers of $CD4^+$ T cells, $CD8^+$ T cells, natural killer cells, and γδ T cells have been found to be decreased in DHF patients compared to those with dengue fever. However, markers of T-cell activation, including elevated levels of soluble CD4 and CD8, IL-2 receptors, and TNF receptors are higher in patients with DHF than with classical dengue fever. Activated $CD4^+$ and $CD8^+$ lymphocytes may themselves also be targets for dengue replication. Apoptosis or immune lysis of dengue-infected monocyte/macrophages cells by $CD4^+$ and $CD8^+$ CTLs results in the release of a variety of vasoactive mediators and procoagulants. IL-10 and secretory TNF-RII are significantly higher in DHF compared to dengue fever patients, and IFNγ levels peak earlier in DHF patients, while IL-6, IL-1r, and macrophage inhibitory factor were higher in fatal DHF cases. The situation is further complicated by evidence of higher levels of secreted NS1 in DHF patients, and complement activation (associated with the NS1 protein) appears to take place at the same time as plasma leakage.

Infection of cell cultures with dengue virus induces production of chemokines, such as IL-8, RANTES, MIP-1α, and MIP-1β, which is consistent with studies in humans. IFN-γ up-regulates both the expression of Fc-γ receptors on monocytes (augmenting the infection of these cells by infectious immune complexes) and the expression of MHC class I and II molecules involved in recognition of these cells by cytotoxic T cells, thereby increasing the potential for enhanced dengue replication and release of cytokine mediators. Activation of complement by virus-antibody complexes or by cytokines released during immune clearance of infected cells is probably also involved in endothelial damage. Elevated levels of TNF-α, IL-6, C3a and C5a, and histamine occur in DHF. TNF-α causes capillary endothelial permeability, and increased plasma levels of this cytokine, in particular, have been associated with severe disease and shock. Dengue virus also infects cells of the mast cell/basophil lineage *in vitro*, suggesting that release of histamine from mast cells may contribute to capillary leakage.

A 43-kDa cytokine ("cytotoxic factor") produced by $CD4^+$ T cells has been demonstrated in sera from DHF patients. When administered to uninfected mice, cytotoxic factor induced vascular and blood-brain barrier permeability and lymphoid cell depletion, mimicking those conditions seen in DHF. Immunization of mice with this cytokine prevented these effects. Although these observations strongly suggest that the disturbances in vascular permeability that account for DHF and DSS are mediated by cytokines, the precise mechanisms remain uncertain and are likely to be complex. Lacking an appropriate animal model, the role of individual cytokines cannot be easily dissected, and the prospects for therapeutic interventions in humans appear to be as, or more, problematic than for bacterial septic shock.

Clinical Manifestations

Illness starts suddenly after an incubation period of 2 to 7 days and can manifest as undifferentiated fever, dengue fever (with or without hemorrhage), or DHF (with or without shock [DSS]).

Undifferentiated Fever. The patient experiences fever with mild nonspecific symptoms that can mimic many acute febrile illnesses. Without specific diagnostic tests, the diagnosis is rarely made since symptoms and physical exam are nonspecific. Patients are typically young children or those experiencing their first infection (primary infection). Full recovery without the need for hospital care is the norm.

Dengue Fever with or without Hemorrhage. Dengue fever begins abruptly with high fever ($\geq 38.5^{o}$ C), chilliness, headache, retrobulbar pain, lumbosacral aching pain, conjunctival congestion, puffiness of the eyelids, gastrointestinal symptoms, and facial flushing. Myalgia, arthralgia, and deep bone pain ("breakbone fever") are characteristic features. Fever may be sustained for as many as 6 to 7 days or may have a biphasic (saddle-back) course. Fever is accompanied by generalized malaise, prostration, anorexia, and nausea. There is no increase in pulse rate related to fever. Respiratory symptoms (cough, sore throat, and rhinitis) are not uncommon, especially in children. A transient, generalized macular or mottled rash may appear on the first or second day. Coincident with defervescence on the third to fifth day, a maculopapular or morbilliform rash appears on the trunk and then on the face and limbs, sparing palms and soles. The rash is nonpruritic, lasts 1 to 5 days, and may result in desquamation on healing. Other signs and symptoms may include generalized lymphadenopathy, cutaneous hyperesthesia, and altered (metallic) taste sensation. Minor hemorrhagic signs are noted in some patients (petechiae, epistaxis). Patients do not develop substantial plasma leak, which is a hallmark of DSS (212). Adults may have a greater propensity for low platelet counts and hemorrhage than children (212).

Myocarditis and various neurologic disorders have been associated with dengue fever. Myocarditis is self-limited and does not result in progressive heart failure. Neurologic manifestations include encephalitis, encephalopathy (without evidence of brain infection), transverse myelitis, peripheral mononeuropathy, polyneuritis, Guillain-Barré syndrome, and Bell's palsy. Reye's syndrome has also been reported to follow dengue infection. Ocular disturbances are rare but include decreased visual acuity, accompanied by retinal hemorrhage, cotton wool exudates, and macular lesions (213). Convalescence may be prolonged, lasting several weeks, with generalized weakness, psychological depression, bradycardia, and ventricular extrasystoles. Persistent arthritis is not a feature of dengue, and there are no known permanent sequelae of classic dengue infection (214). Hemophagocytic syndrome has been reported in association with dengue fever and dengue shock syndrome (215, 216).

Maternal dengue infection generally results in a normal fetal outcome (217, 218); congenital abnormalities have not been definitively linked to dengue infection. Fetal deaths have been recorded during acute maternal infection, but causality could not be determined. Maternal infection near the time of parturition has been reported to result in hemorrhagic complications and premature delivery (219), as well as transplacental infection and severe dengue in the neonatal period (220), but such events are rare.

The similar clinical presentation of dengue fever and DHF creates difficulties in differentiating them early in the course of illness. Close monitoring, such as monitoring fever, fluid intake, and the presence of warning signs (abdominal pain, persistent vomiting, clinical fluid accumulation, mucosal bleed, lethargy, liver enlargement, increasing hematocrit, decreasing platelet count), is necessary around the time of defervescence so that early and appropriate therapy for DHF/DSS can be initiated.

DHF. This clinical condition can be characterized by three phases: (i) the febrile phase, (ii) the critical (plasma leak) phase, and (iii) the convalescent (reabsorption) phase.

During the early febrile phase, which typically lasts 2 to 7 days, patients with DHF can present with symptoms much like those of dengue fever, but they may also have hepatomegaly without jaundice (later in the febrile phase). The hemorrhagic manifestations that occur in the early course of DHF most frequently consist of mild hemorrhagic manifestations as in dengue fever. Less commonly, epistaxis, bleeding of the gums, or frank gastrointestinal bleeding occurs while the patient is still febrile.

The critical (plasma leak) phase, which usually lasts 24 to 48 hours, begins about the time fever abates. At this time, it is vital to watch for evidence of hemorrhage and plasma leak into the pleural and abdominal cavities and to implement appropriate therapies replacing intravascular losses and stabilizing effective volume. If left untreated, this can lead to intravascular volume depletion and cardiovascular compromise. Evidence of plasma leak includes sudden increase in hematocrit ($\geq 20\%$ increase from baseline), presence of ascites, a new pleural effusion on lateral decubitus chest x-ray, or low serum albumin or protein for the patient's age and sex. Patients with plasma leak should be monitored for early changes in hemodynamic parameters consistent with compensated shock, such as tachycardia, especially in the absence of fever, weak and thready pulse, cool extremities, narrowing pulse pressure (systolic minus diastolic blood pressure < 20 mmHg), delayed capillary refill (> 2 seconds), and oliguria. Once frank shock is diagnosed, the patient is categorized as having DSS. Prolonged shock is the main factor, associated with complications, that can lead to death, including massive gastrointestinal hemorrhage. Interestingly, many patients with DHF/DSS remain alert and lucid throughout the course of the illness, even at the tipping point of profound shock.

Indicators that a patient has entered the critical phase include sudden change from high to normal or subnormal temperature, rapid decrease in platelet count, and hemoconcentration (hematocrit increased by $> 20\%$), new hypoalbuminemia or hypercholesterolemia, new pleural effusion or ascites, and signs and symptoms of impending or frank shock.

During the recovery phase, which typically lasts 48 to 72 hours, a gradual reabsorption of extravascular compartment fluid takes place. The hematocrit stabilizes or may be lower due to the dilutional effect of reabsorbed fluid. Congestive heart failure and respiratory distress from pleural effusion and ascites may occur if excessive fluids have been administered.

Dengue Case Classification. The 1997 WHO dengue case definition was limited in terms of complexity and applicability. According to the new case definitions adopted in 2009, clinical dengue infection is classified as (i) dengue without warning signs, (ii) dengue with warning signs, and (iii) severe dengue (Table 5).

TABLE 5 Clinical description of the 2009 WHO revised dengue case definitions

Dengue without warning signs
Fever and two of the following:
Nausea, vomiting
Rash
Aches and pains
Leukopenia
Positive tourniquet test
Dengue with warning signs
Dengue as defined above with any of the following:
Abdominal pain or tenderness
Persistent vomiting
Clinical fluid accumulations (ascites, pleural effusion)
Mucosal bleeding
Lethargy, restlessness
Liver enlargement >2 cm
Increase in hematocrit concurrent with rapid decrease in platelet count
Severe dengue
Dengue with at least one of the following:
Severe plasma leakage leading to
Shock (DSS)
Fluid accumulation with respiratory distress
Severe bleeding as evaluated by a clinician
Severe organ involvement:
Liver: AST or ALT ≥1000
CNS: impaired consciousness
Failure of heart or other organs

Diagnosis

Diagnosis depends on the consideration of clinical features and potential exposure, based on residence or travel in dengue-endemic areas, and knowledge about the occurrence of other cases in the community. The disease may resemble influenza, rubella, rubeola, malaria, scrub typhus, leptospirosis, and a variety of other arbovirus infections, especially chikungunya, O'nyong nyong, West Nile, Sindbis, Mayaro, Edge Hill, Kokobera, Spondweni, Barmah Forest, Zika, and Ross River virus diseases. With its rising incidence in tropical areas affected by dengue, leptospirosis, in particular, has caused considerable diagnostic confusion with dengue.

The approach to laboratory diagnosis depends on the timing of sample collection relative to illness onset. Viremia is detectable via culture, PCR, and antigen detection from blood during the first 3 to 5 days of illness before defervescence. *A. albopictus* C6/36 mosquito cell lines are the preferred viral isolation system, but mosquito inoculation is more sensitive. In practice, however, virus isolation is not practical for routine diagnosis, since it requires cell culture facilities and has a long turnaround time and lower sensitivity compared to molecular or immunoassay methods (221).

RT-PCR with type-specific primers is useful for serotype-specific diagnosis. Multiplex RT-PCR can provide a method for detection and typing multiple dengue viruses in clinical samples or cell culture fluids. Viral nucleic acid can be detected in serum, plasma, blood (including that collected on filter paper), saliva, and tissues (including formalin fixed). Many RT-PCR protocols are available; however, many have not been validated. Several manufacturers offer dengue RT-PCR kits, but an FDA-approved real-time RT-PCR kit available from the CDC allows viral detection in the first 7 days of illness (222).

NS1 antigen can be detected in blood, for as long as 9 days after fever onset, and in tissue samples. Commercial ELISA and rapid tests are available in some countries. These tests are particularly attractive since they can detect dengue virus early during the course of illness when accurate diagnosis is most clinically relevant, have rapid turnaround, and can be conducted in resource-poor settings without specialized equipment for nucleic acid detection. Rapid tests that can detect both NS1 antigen and IgM antibody are also available. In a multicenter study the NS1 ELISA sensitivity was 60 to 75% and specificity was 71 to 80%, while NS1 rapid diagnostic test sensitivity was 38 to 71% and specificity was 76 to 80% (223). Sensitivities were generally higher in primary than secondary dengue infections.

Definitive serologic diagnosis depends on the demonstration of a 4-fold or greater rise (or fall) in antibody titers. The IgM antibody-capture ELISA is a useful diagnostic test, and a positive result on a single serum sample provides a presumptive diagnosis. IgM antibodies appear shortly after defervescence (on days 4 to 6 after onset), are detectable in 50% of patients by 3 to 5 days of illness and in 99% by day 10, and wane after 1 to 2 months. IgM antibodies are found following both primary and secondary infections; however, in secondary infection early convalescent stage IgM levels are significantly lower than in primary infections and may be undetectable in some instances (224). A negative IgM test before the sixth day of onset is not definitive, whereas a negative result after that day suggests another etiology. A small proportion of cases of secondary infection will have no detectable IgM response.

The IgM antibody-capture ELISA is the most common method of serologic diagnosis and can be applied to serum, blood on filter paper, and saliva. In addition, rapid tests for detection of IgM antibody are available. In a multicenter study the IgM ELISA sensitivity was 96 to 98% and specificity was 78 to 91%, while IgM rapid diagnostic test sensitivity was 30 to 96% and specificity was 86 to 92% (223).

The HI, CF, IgG, ELISA, and neutralization tests are useful for diagnosis of recent infection. Preferably an acute sample is obtained as early as possible (in the febrile phase) and a second specimen is obtained 2 to 3 weeks later. Serotype-specific diagnosis by a ≥4-fold rise in antibody titer is relatively simple in the case of primary infections, but broad cross-reactions create considerable difficulties in serotype-specific diagnosis in cases of secondary infections. The plaque reduction neutralization test, which is more specific than other tests, or epitope-blocking ELISAs employing monoclonal antibodies, may help to distinguish specific from cross-reactive antibody responses. In the case of sequential dengue infections, the antibody response to the initial infecting virus type may exceed that of the current infecting type ("original antigenic sin"). Secondary dengue infections are characterized by the presence of HI antibodies at titers greater than 20 in the acute phase sample and by high titers (>1,280) in convalescent sera. The ratio of IgM and IgG antibodies determined by ELISA has been used to distinguish primary from secondary infection; in primary infections the IgM/IgG ratio in acute sera or convalescent sera obtained during the first month after onset generally exceeds 1.5, whereas secondary infections are characterized by an excess of IgG (225).

Prevention

Prevention of dengue transmission by control or eradication of *A. aegypti* by elimination of breeding sites in contemporary settings has been successful only in a few locales (e.g., Singapore and Cuba) where intensive efforts have been sustainable. However, even in Singapore, dengue has resurged in recent years as a result of decreased herd immunity, virus transmission outside the home, an increase in the age of infection, and adoption of a case-reactive approach to vector control (226). One model, that estimated the *Ae. aegypti* threshold density (expressed as the standing crop of pupae per human inhabitant) required for virus transmission, found that the threshold levels ranged between 0.5 and 1.5, thus illustrating the extreme difficulty in dengue prevention by means of vector-source reduction (227). Unfortunately, epidemic control by use of aerial or ground applications of mosquito adulticides has not been highly successful, in part because *Ae. aegypti* resting places are not reached by aerosols.

New approaches to *A. aegypti* control in development include lethal ovitraps, release of *Wolbachia*-infected mosquitoes, release of male mosquitoes carrying a dominant lethal gene (RIDL), and use of insecticide-treated curtains (228–230). While many of these methods show promise in small-scale field trials, studies have not been conducted on a scale capable of demonstrating reduction in dengue incidence. Epidemiologic investigations of dengue outbreaks on the Texas-Mexico border suggest that the use of air conditioning strongly protects inhabitants from dengue infection (231).

The long-term solution to the control of dengue is the wide-scale use of safe, effective, and inexpensive vaccines against all four dengue serotypes. Challenges to dengue vaccine development include the need for protection against all four serotypes, the possibility that vaccine immunity might predispose to immunopathological events associated with DHF, the lack of understanding of the pathophysiology of infection, and the lack of suitable animal models. Many approaches to dengue vaccines are currently under investigation, including live attenuated, chimeric, DNA, subunit, and inactivated vaccines (232).

A recombinant, live attenuated tetravalent chimeric vaccine (Sanofi Pasteur) has been evaluated in two phase-3 randomized clinical trials in children between the ages of 2 and 14 years in the Asia-Pacific region and between the ages of 9 and 16 years in Latin America (233, 234). This 3-dose (0, 6, and 12 months) vaccine is based on the yellow fever 17D vaccine virus backbone, substituting the dengue envelope genes from each of the four dengue serotypes. The vaccine had a 57% efficacy in the Asia-Pacific trial and a 61% efficacy in the Latin American trial. Efficacy varied among the dengue serotypes, with particularly low efficacy noted for the dengue 2 serotype and among younger children. No evident vaccine safety issues were noted in either trial. In a subsequent 3-year follow-up of children enrolled in these two trials, as well as an earlier phase 2b trial in Thailand, vaccinated children aged 9 years and older at baseline had a lower risk of dengue-related hospitalization (relative risk=0.50, 95% CI: 0.29–0.86) while younger vaccinated children had a higher risk of hospitalization (relative risk=1.58, 95% CI: 0.83–3.02) (235). An overall efficacy of 60% for prevention of symptomatic dengue was similar to that noted in earlier analysis, with higher efficacy noted in older children (66% compared to 45% in younger children). This vaccine is now licensed in several countries (Brazil, El Salvador, Mexico, and the Philippines as of February 2016).

Two other dengue vaccines currently in clinical development demonstrate adequate immunogenicity (i.e., seroconversion demonstrated as a neutralization titer of 1 in 10 or a 4-fold increase in neutralization titers). A 2-dose, live attenuated tetravalent chimeric vaccine (Takeda) engineered the attenuated dengue 2 PDK-53 vaccine strain developed by Mahidol University as a backbone by replacing the dengue serotype 2 virus prM and E structural proteins with the comparable proteins of dengue serotypes 1, 3, and 4. It was well tolerated and immunogenic in phase-1 trials (236). A live attenuated tetravalent vaccine developed at the National Institutes of Health is based on wild-type serotype-1 and -4 viruses, each with an attenuating 30-nucleotide deletion (Δ30) in the 3′ untranslated region (rDEN1Δ30 and rDEN4Δ30). The vaccine for the dengue 2 (rDEN2/4Δ3[prME]) component uses the dengue 4 vaccine strain (rDEN4Δ30) backbone and the premembrane and envelope proteins of dengue 2. The genotype 3 vaccine (rDEN3Δ30/31) component is based on a dengue 3 virus with a 30-nucleotide deletion similar to the other strains with an additional 31-nucleotide deletion upstream of the Δ30 mutation. A single dose of this vaccine produced seroconversion rates of 92% or greater for all four dengue genotypes and was well tolerated (237).

Treatment

The WHO updated its guidelines for the management of dengue in 2009. Initial assessment, including patient history and a full blood count, should determine the dengue phase (febrile, critical, or recovery), whether warning signs (Table 5) are present, hydration and hemodynamic status, and whether the patient requires admission. Ambulatory patients should be reviewed daily for disease progression until they are out of the critical period around defervescence. Uncomplicated dengue fever treatment is supportive and includes bed rest, fluid replacement, antipyretics, and analgesics. Aspirin or nonsteroidal anti-inflammatory agents should be avoided due to the hemorrhagic diathesis and a possible relationship between dengue and Reye's syndrome. Patients with warning signs, or those with co-existing conditions that may complicate dengue treatment, should be hospitalized for observation and fluid management. Patients with evidence of severe plasma leakage, hemorrhage, or organ impairment (hepatic damage, renal impairment, cardiomyopathy, encephalopathy, or encephalitis) require emergency treatment. For these patients, judicious intravenous fluid resuscitation is the essential, and usually sole, intervention required. Detailed algorithms for fluid management are provided in the WHO guidelines. Fluid overload, which will produce pleural effusions, ascites, pulmonary edema, and irreversible shock (heart failure, if in combination with ongoing hypovolemia) should be avoided. Blood transfusion is lifesaving for severe bleeding; however, care must be given to avoid fluid overload. Other aspects of treatment involve monitoring for hyperglycemia or hypoglycemia and electrolyte and acid-base imbalance. Co-infections, such as malaria, typhus, and leptospirosis, should also be considered. Prompt recognition and treatment of DSS can reduce mortality to less than 1% (238–241). Corticosteroids, chloroquine, lovastatin, and the polymerase inhibitor belapiravir have not proven effective in controlled clinical trials (238–240, 242).

There are intense efforts to develop antiviral drugs for dengue. Most are based on structure-function studies that are identifying drug targets. Most focus on the protease, RNA-dependent RNA polymerase, or NS4B. Many show antiviral

activity *in vitro* (162, 243–249) and to date only a few show activity in small animal models. The most advanced was NITD-0008; however, this had toxicity issues and development was discontinued (250). Prochlorperazine, a dopamine D2-receptor antagonist approved for treating nausea and emesis, is inhibitory for dengue replication *in vitro* and in *Stat1*-deficient mice (251). None of these agents has advanced to clinical evaluation.

Zika Fever

Zika fever is caused by Zika virus, which was first isolated from a rhesus monkey in the Zika Forest, Uganda, in 1947. The virus is thought to exist in an *Aedes* mosquito—monkey cycle. Until recently very few clinical cases of Zika fever were recorded, and the virus was considered to cause sporadic infections in countries in Africa and Asia. However, in 2007, a Zika virus outbreak on Yap Island, Federated States of Micronesia, in the Pacific, involving 108 confirmed cases and a further 72 suspected cases (252), represented the first documented transmission outside of endemic areas in Africa and Asia. Subsequently, the virus was found in the Philippines (2012) and French Polynesia (2013–2014), with subsequent spread across the South Pacific (New Caledonia, Easter Island, and the Cook Islands). During 2015 the virus has greatly expanded its geographic range into the Americas with confirmed reports from Brazil in May 2015 onwards (253, 254), followed by reports from a further 26 countries in the Americas during the second half of 2015 and into early 2016. Zika fever is now considered an important emerging disease, and WHO declared the outbreak to be a Public Health Emergency of International Concern on 1 February 2016 (255). An updated review of the virus has recently been published (256).

The virus is spread by the bite of *Aedes* mosquito species and approximately 20% of infections lead to clinical disease. The incubation period is 3 to 12 days. Clinical symptoms are usually mild, often consisting of fever, rash, joint pain, and red eye, and sometimes muscle pain, headache, retro-orbital pain, and vomiting. Symptoms last up to one week. Hospitalization is rare, and fatalities are infrequent. Sexual transmission has occurred, and virus has been detected for several weeks in semen, sometimes in association with hematospermia (257, 258). Transfusion-mediated transmission appears possible, as 3% of blood donors were RT-PCR positive for Zika virus during the French Polynesia outbreak (259). The French Polynesian epidemic included cases of GBS that are still being investigated (260). While the causal link between Zika virus infection and GBS remains to be confirmed, multiple countries in the Americas have reported increased GBS case numbers in the context of the current outbreak.

Until 2015 there has been one case of virus transmitted to a fetus during the third trimester and one case potentially acquired via sexual contact. However, during 2015 the Brazilian Ministry of Health reported an epidemic of congenital microcephaly (a description of a condition where the occipital frontal circumference of the head of a newborn child is less than that of the normal circumference for gestational age, sex, and race) associated with Zika virus infection. Other reported abnormalities include intracranial calcifications, ventricular dilation, macular lesions, and hearing loss (261, 262). As of January 30, 2016, the Brazilian Ministry of health had reported 4,783 cases of microcephaly or central nervous system malformation, including 476 deaths, in at least 20 states of Brazil. At present, the relationship between Zika virus infection and congenital microcephaly is primarily ecological, and investigation of the causal link between Zika virus and microcephaly is ongoing (263). However, there are reports of detection of viral RNA by RT-PCR in amniotic fluid samples from pregnant women whose fetuses had microcephaly (264) and detection of viral RNA in blood and tissue samples from a newborn baby with congenital abnormalities. In addition, there has been a recent report of characterization of Zika virus–associated cases of microcephaly (265).

There is no vaccine, and treatment is supportive. Guidances with regard to infection prevention, including sexual transmission, and evaluation of potentially affected infants and exposed women are available on US CDC (266, 267) and WHO websites.

Other flaviviruses associated with rare and sporadic human disease are listed in Table 4.

Flaviviruses Causing the Hemorrhagic Fever Syndrome

Dengue Viruses

Dengue viruses (discussed above) are a leading cause of hemorrhagic fever throughout the tropics.

Yellow Fever Virus

In 1901 Major Walter Reed, a US Army physician, and colleagues, working in Cuba, proved that yellow fever was transmitted by the bite of an *Aedes aegypti* mosquito rather than by physical contact of clothing or bedding from infected individuals. This groundbreaking research led to studies on epidemiology of infectious diseases. Subsequently, in 1927, Sawyer and colleagues isolated yellow fever virus from a Ghanan named Asibi by passage of Asibi's serum in monkeys. Yellow fever virus, the prototype member of the family *Flaviviridae*, causes a hemorrhagic fever syndrome characterized by high viremia; hepatic, renal, and myocardial injury; hemorrhage; and high lethality.

Biology

Nucleotide sequencing has placed yellow fever virus within a group of eight other viruses in the *Flavivirus* genus, including Banzi, Edge Hill, Entebbe bat, Jugra, Sepik, Uganda S and Wesselsbron, and Yokose. Analyses of the yellow fever virus nucleotide sequences have distinguished at least 7 genotypes: two in West Africa, with probable subdivisions between western (Senegal) and eastern (Nigeria) strains; one in Central and East Africa; one in East Africa; one in Angola; and two in South America. South American genotype I is present in Brazil, Colombia, and Ecuador, whereas genotype II is found in Peru and Bolivia. Genotype II exhibits a higher degree of microheterogeneity, and a phylogenetic pattern suggesting independent evolution in localized geographic areas (268). Phylogenetic studies of the West African and South American genotype-I strains show genetic relatedness, supporting the notion that yellow fever virus was introduced into the New World from West Africa during the slave trade. Homology across all yellow fever genotypes is >90%, suggesting that the virus is relatively resistant to mutational change either because of gene flow within large geographic regions or because of restrictions on change based on the requirement for specific vectors and vertebrate hosts.

The host range of yellow fever is shown in Table 3. *Aedes pseudoscutellaris* (AP-61) mosquito cells are highly susceptible, with virus replication assessed by CPE, immunofluorescence, or subpassage to mice or mammalian cells.

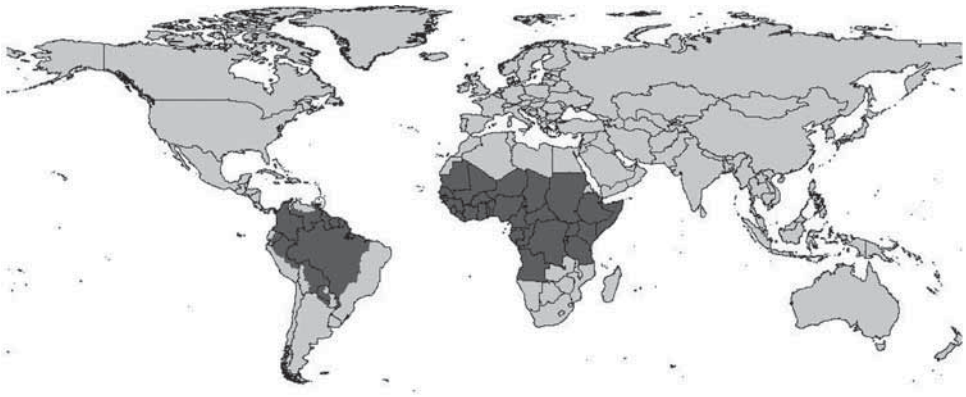

FIGURE 11 Geographical distribution of yellow fever.

Intrathoracic inoculation of mosquitoes (*Toxorhynchites* or *A. aegypti*) is also a sensitive method for isolation or assay of yellow fever virus. Asian (rhesus and cynomolgus) monkeys, as well as some New World monkeys infected with yellow fever virus, develop fatal hepatitis resembling the human disease, whereas African species, with which the virus has coevolved, are resistant to overt disease.

Epidemiology

Distribution and incidence. Yellow fever occurs only in tropical South America (13 countries), and sub-Saharan Africa (31 countries) (Fig. 11). Yellow fever has never invaded Asia. Possible explanations include (i) cross-protection afforded by dengue immunity and (ii) low vector competence of Asian strains of *A. aegypti* (there is experimental evidence supporting both hypotheses); (iii) occurrence of yellow fever in remote areas; and (iv) exclusion of infected persons from routes of travel. In Africa, where approximately 90% of cases occur, there were an estimated 130,000 (95% CI 51,000–380,000) cases with fever and jaundice or hemorrhage, including 78,000 (95% CI 19,000–180,000) deaths in 2013 (269). In South America, yellow fever occurs in the Amazon, Orinoco, and Araguaia River basins and contiguous areas, and, intermittently, on the island of Trinidad.

The incidence of yellow fever in South America is highest during months with peak rainfall, humidity, and temperature (January to March). In tropical America, jungle yellow fever principally affects young adult males exposed, during forest-clearing activities, to the vector (*Haemagogus* spp.), which inhabits the rain forest canopy. In Africa, transmission peaks during the late rainy season and early dry season. A wider array of vectors is responsible for transmission, and they reach highest densities in savanna habitats. Human infection is endemic, the prevalence of immunity increases with age, and therefore children are at greater risk. There is little difference in incidence by sex.

During epidemics in Africa, the incidence of infection may be as high as 20%, and the incidence of disease may be 3% (270). The infection/case ratio ranges between 2:1 and 20:1, respectively. Immunity to certain heterologous flaviviruses ameliorates the disease and increases this ratio. The case-fatality ratio has varied widely in different epidemics, possibly reflecting virus-strain variation in virulence or sensitivity of case detection. Rates higher than 50% are reported, but the lethality of yellow fever with jaundice usually approximates 20%.

Transmission. The virus is transmitted between wild nonhuman primates and diurnally active mosquitoes that breed in tree holes (*Haemagogus spp.* in the Americas, and *Ae. africanus* in Africa) (271) (Fig. 10). Humans are infected when they are exposed to these vectors ("jungle yellow fever"), and epidemic-spread from human to human can be maintained in rural areas by the same mosquitoes. Alternatively, *A. aegypti*, a domestic mosquito that breeds in manmade containers, may transmit yellow fever virus between humans as the sole viremic hosts in this cycle ("urban yellow fever"). In the moist savanna region of Africa, tree-hole-breeding *Aedes* spp. (e.g., *Aedes furcifer* and *Aedes luteocephalus)* are implicated in endemic and epidemic transmission. The virus is maintained over the dry season by vertical transmission in mosquitoes.

Pathogenesis

The pathophysiology of yellow fever disease is multifactorial, involving direct viral injury, necrotic and apoptotic processes in virus-infected hepatocytes, hypoxia, and induction of a pro-inflammatory innate immune response. Signs of specific organ dysfunction (hepatitis and renal injury) follow onset of illness by several days. The virus initially replicates in regional lymph nodes and then spreads via the bloodstream to other tissues, including liver, spleen, bone marrow, and myocardium. In liver, Kupffer cells are infected first, followed by hepatocytes in the midzone (zone 2) of the liver lobule, which undergo coagulative necrosis, sparing cells bordering the central vein and portal tracts. The reason for this peculiar distribution of hepatic injury is unknown. However, midzonal necrosis has been described in low-flow hypoxia, due to ATP depletion and oxidative stress of marginally oxygenated cells at the border between anoxic and normoxic cells. Yellow fever virus antigen has been observed principally in hepatocytes in the midzone, suggesting a predilection of these cells to virus replication. Injury to hepatocytes is characterized by eosinophilic degeneration (Councilman bodies), rather than by ballooning and rarefaction necrosis typically seen in virus hepatitis. Cytopathic changes (including Councilman bodies) are the result of programmed cell death (apoptosis), which is the dominant mechanism of cell injury in the liver (272). Consistent with apoptotic cell death, only minimal mononuclear inflammation develops in yellow fever. Other hepatic pathologic changes include microvesicular accumulation of fat, ceroid/lipofucsin deposits, and intranuclear (Torres) bodies. The reticulin framework is preserved, and healing occurs without cirrhosis. Renal pathology is

characterized principally by acute tubular necrosis and fatty change, which may represent late-stage injury following shock. Cerebral edema and petechial hemorrhages are common findings. Lymphocytic elements in the spleen and lymph nodes are depleted, and large mononuclear or histiocytic cells accumulate in the splenic follicles.

In addition to hepatic and renal dysfunction, the disease is characterized by hemorrhage and circulatory collapse. Decreased synthesis of vitamin K–dependent coagulation factors by the liver, disseminated intravascular coagulation, and altered platelet function contribute to the hemorrhagic diathesis (273). Direct virus injury to myocardial fibers, which show cloudy swelling and fatty changes, may contribute to shock. However, shock is undoubtedly mediated by cytokine dysregulation. TNF-α, IFN- γ, TGF-β, and inducible nitric oxide produced by infected/activated Kupffer cells, and splenic macrophages might play a prominent role in cell injury, oxygen radical formation, microvascular damage and microthrombosis, tissue anoxia, and shock. Patients dying of yellow fever show cerebral edema at autopsy, probably the result of microvascular dysfunction.

Yellow fever infection is followed by a rapid immune response. Neutralizing antibodies, cytolytic antibodies against viral proteins on the surface of infected cells, antibody-dependent cell-mediated cytotoxicity, and cytotoxic T cells are presumed to mediate the clearance of primary infection (274). Neutralizing antibodies appear toward the end of the first week after onset, and it is notable that antibody (and presumably cellular) responses occur coincident with the clinical crises; however, it is unclear whether immune mechanisms during the acute stage of disease contribute to pathogenesis. Neutralizing antibodies persist lifelong after yellow fever infection, and provide complete protection against disease on re-exposure to the virus.

Clinical Manifestations

The incubation period from the bite of an infected mosquito to onset of fever is 3 to 6 days. The clinical expression of disease varies from nonspecific influenza-like illness to life-threatening hemorrhagic fever (274). Onset is abrupt, with fever, chills, malaise, headache, lumbosacral pain, generalized myalgia, nausea, and dizziness. The patient appears toxic, with congestion of the conjunctivae and face, a furred tongue with reddening of the edges, and a relative bradycardia in the face of fever (Faget's sign). Leukopenia with a relative neutropenia is typically present. This "period of infection," during which the patient is viremic and may serve as a source of infection for blood-feeding mosquitoes, lasts up to several days. It is sometimes followed by a distinct "period of remission" with abatement of fever and symptoms lasting 2 to 24 hours. In the abortive case, the patient may simply recover, without further signs or symptoms. In other cases, the illness reappears with fever, nausea, vomiting, epigastric pain, and the onset of jaundice, renal dysfunction, and a hemorrhagic diathesis. During this "period of intoxication," viremia is usually absent. Over the course of 3 to 5 days, jaundice and serum transaminase levels increase. The direct bilirubin level reaches levels between 10 and 15 mg/dL. There is a marked increase in albuminuria, a reduction in urine output, and rising azotemia. Albumin levels in the urine range between 3 and 20 g/L. Variable hemorrhagic phenomena occur, including coffee-grounds hematemesis, melena, metrorrhagia, petechiae, ecchymoses, epistaxis, oozing of blood from the gums, and excessive bleeding at needle-puncture sites. Laboratory abnormalities include thrombocytopenia, prolonged clotting and prothrombin times, and global reductions in clotting factors. Some patients appear to have a pattern of clotting abnormalities, suggesting disseminated intravascular coagulation. Death occurs on the seventh to tenth day of illness. Preterminal events include hypothermia, agitated delirium, intractable hiccups, hypoglycemia, hyperkalemia, stupor, and coma. The electrocardiogram may show ST-T wave abnormalities. CSF is under increased pressure, with elevated albumin, but contains no increase in cells, findings that are consistent with cerebral edema.

Convalescence may be associated with weakness and fatigability lasting several weeks. Late deaths have occurred weeks after the illness and have been ascribed to cardiac arrhythmia. The duration of icterus in surviving cases is unknown. Elevations of serum transaminase levels have been documented to persist for at least 2 months after onset of yellow fever. The hepatitis resolves eventually without postnecrotic scarring.

Diagnosis

Diseases that must be differentiated clinically from yellow fever include viral hepatitis, leptospirosis, dengue hemorrhagic fever, Rift Valley fever, Congo-Crimean hemorrhagic fever, severe malaria, Q fever, and typhoid fever. Other virus hemorrhagic fevers, including Lassa, Marburg, and Ebola virus diseases, Bolivian, and Argentine hemorrhagic fevers, are not usually associated with jaundice. The isolated case of yellow fever obviously presents a more difficult diagnostic challenge than do a cluster of similar cases. However, outbreaks of leptospirosis and hepatitis E (with deaths in pregnant women) and delta hepatitis have been confused with yellow fever in the past. The high levels of protein in the urine help distinguish yellow fever from severe malaria (blackwater fever).

The virus is most readily isolated from blood during the first 4 days after onset; it is occasionally isolated at later times. The virus may also be recovered from postmortem liver tissue. However, hepatic biopsy during the illness is contraindicated and has led to fatal hemorrhage in patients in whom this procedure was performed. Virus is isolated by intracerebral inoculation of mice, intrathoracic inoculation of *Toxorhynchites* mosquitoes, or inoculation of mosquito cell cultures, particularly *A. pseudoscutellaris* cells, with detection of virus after 3 to 4 days by immunofluorescence or RT-PCR.

Viral antigen is detectable in serum by immunoassay in a high proportion of cases, and the test may detect noninfectious antigen in poorly handled specimens. RT-PCR may also be used for rapid detection of virus nucleic acid in blood. A definitive postmortem diagnosis is made by detection of yellow fever antigen in liver tissue sections by immunocytochemical staining or RT-PCR. There are no currently available commercial diagnostic tests; the assays described remain special procedures in research and reference laboratories.

The presence of IgM antibodies in a single sample provides a presumptive diagnosis, and confirmation is made by a rise in titer between paired acute- and convalescent-phase samples. Although older methods for serologic methods for diagnosis (HI, CF, indirect immunofluorescence, and neutralization tests) are useful procedures, they have largely been replaced by the ELISA, particularly the IgM capture immunoassay.

Prevention

The control of *A. aegypti* is discussed above for dengue virus. The control of yellow fever epidemics involving wild vector species is more difficult. Aerial applications of ULV insecticides have been attempted in the past.

The risk of acquiring yellow fever illness among unvaccinated travelers to West Africa during the peak transmission season from July to October was estimated at 50 per 100,000 for a 2-week stay. Measures to avoid mosquito bites are likely to provide incomplete protection, making immunization the best preventive strategy. A valid certificate of vaccination is required under the International Health Regulations for entry into yellow fever endemic countries and for travel from such countries to receptive (*A. aegypti*-infested) countries. Detailed information on these requirements can be obtained from the CDC website.

Yellow fever 17D is a highly effective, generally safe, live attenuated vaccine. Protective immunity (conferred by neutralizing antibodies) occurs in >90% of vaccinees within 10 days and 99% in 30 days after vaccination, and probably provides lifelong protection after a single dose (275). Until recently, booster vaccinations were required every 10 years to maintain protective immunity; however, in June 2013 the World Health Organization recommended that a single dose of yellow fever vaccine was sufficient to induce lifelong protective immunity against yellow fever in healthy individuals (276). Subsequently, in 2015, the Advisory Committee on Immunization Practices (ACIP) concurred that a single dose of yellow fever vaccine provides long-lasting protection and is adequate for most travelers, and, in addition, approved recommendations for certain categories of travelers to receive additional doses of yellow fever vaccine (277).

The vaccine may be simultaneously administered with other vaccines used for childhood immunization (measles, polio, DPT, hepatitis B) or travel (hepatitis A, oral cholera, oral or parenteral typhoid). In practice, few vaccinees complain of local injection inflammation, fever, mild headache, myalgia, and malaise. In clinical trials, where symptoms have been prompted, these reactions are more frequent but are mild and do not interfere with activities. Severe or serious adverse reactions to 17D vaccine are extremely rare. Although over 500 million persons have received the vaccine, there have been 218 cases of vaccine-associated neurologic disease, of which a 3-year-old child in the US and 53-year-old HIV-positive man in Thailand died. Virus recovered from the brain of the child who died contained two amino acid changes in the E gene and exhibited increased neurovirulence in animals (278) suggesting that mutation of the vaccine virus during replication in the patient was responsible for the vaccine accident. The vaccine is thus not recommended for infants younger than 9 months (6 months during epidemic risk) and is contraindicated for infants younger than 5 months.

In an analysis of post-marketing safety data in the US, a higher incidence of severe adverse reactions was noted in elderly persons, with those >75 years having a risk 12 times higher than young adults. The adverse experiences were not well characterized and included multisystem and neurological incidents. Three deaths in elderly persons with multisystem disease, including liver and kidney involvement and shock, occurred in the US in the late 1990s (279) and were linked to yellow fever vaccine; two deaths also occurred in Brazil (280). Pathological examination and case histories in these fatal cases of viscerotropic disease were similar to infection with wild-type yellow fever virus, however, were caused by vaccine virus with no detectable mutations that could explain the wild-type-like clinical disease. To date there have been 72 reported cases of vaccine-associated viscerotropic disease with a case-fatality rate of approximately 50%. All were first-time vaccinees, showed clinical signs of disease within 2 to 5 days of immunization, and were 4 to 79 years old, with most aged over 60 years. Although extremely rare, it appears that yellow fever vaccine can cause viscerotropic fatal infections resembling yellow fever disease. Evidence to date suggests that this is not due directly to the vaccine virus; rather it appears to be an atypical host response to the vaccine.

The vaccine is manufactured in embryonated chicken eggs and is contraindicated for persons with a history of egg allergy (e.g., oral intolerance to eggs). Travelers who require immunization but who have a history of egg allergy should be skin-tested as directed in the vaccine label; in case a reaction to the vaccine occurs, the full dose should not be administered and a neutralization test should be performed 10 to 14 days later to determine if the patient has seroconverted as a result of the low (skin-test) dose of virus. If seroconversion has occurred, the patient may be reassured that she is protected against natural infection. If not, desensitization before vaccination may be considered (as described in the product label), or, alternatively, personal protection against mosquito bite or avoidance of travel may be recommended. The physician faced with questions of allergy (or other contraindications) should carefully consider the true risk of exposure based on the regions to be visited and may wish to consult an expert in the epidemiology of yellow fever. In persons without egg allergy, systemic allergic reactions occur at a very low rate (approximately 1:131,000 [281]) and may be due to sensitivity to gelatin used to stabilize the vaccine.

The safety of yellow fever vaccination during pregnancy has not been clearly established. In limited studies, congenital infection appears to occur at a low rate (probably 1 to 2%) and has not been clearly associated with fetal abnormalities. An underpowered case-control study of vaccination in early pregnancy indicated a relative risk of spontaneous abortion of 2.3 (282). In another (uncontrolled) analysis (283), the authors concluded that, while spontaneous abortion and congenital anomalies could not be excluded as effects of the vaccine, there was no medical rationale to interrupt pregnancy if inadvertent vaccination in pregnancy is performed. Pregnant women may be reassured that there is no risk to themselves and very low (if any) risk to the fetus from the vaccine. Patients should be followed to determine the outcome of pregnancy, and, if fetal abnormality is noted, cord blood should be obtained for an IgM test to determine whether congenital infection has occurred.

The immune response to yellow fever vaccination during pregnancy is impaired, and revaccination is indicated after parturition (284). Other factors that may impair seroconversion to 17D vaccine include malnutrition, simultaneous administration of injected cholera vaccine, and HIV infection. Asymptomatic HIV-infected travelers with CD4+ cell counts >200/mm^3, who require the vaccine, should be immunized, but it may be prudent to confirm that they have developed neutralizing antibodies because two studies have shown lower rates of yellow fever virus–specific neutralizing antibodies among HIV-infected persons compared with uninfected controls at 10 to 12 months postvaccination (285, 286). Adverse events do not appear more frequently in HIV-infected subjects, although one case of vaccine-associated neurological disease occurred in an HIV-infected man with a low CD4+ count (108/mm^3). Immunosuppressed persons who are unable to effectively resist viral infections should not be vaccinated. A history of thymus disease is a contraindication to yellow fever vaccine.

Treatment

Treatment of yellow fever is symptomatic (273). There is little experience in the management of patients in modern intensive-care settings. Salicylates should be avoided due to the hemorrhagic diathesis. On theoretical grounds, severely ill patients should benefit from supplemental oxygen, fluid and electrolyte management, and circulatory support. In cases with severe hemorrhage, blood replacement may be indicated. ATP depletion of the liver might be countered by administration of glycolytic substrates (fructose). Heparin treatment to reverse disseminated intravascular coagulation has been proposed but should be considered only in cases that have been extensively evaluated and in which consumption of clotting factors and activation of fibrinolytic mechanisms appear to predominate over diminished production. Hemodialysis may be indicated in patients with severe renal impairment. No effective antivirals are currently available, but favipiravir and BCX4430 show activity in animal models (287, 288) and would be candidates for study based on studies in a hamster model. Ivermectin and imidazole-4,5- and pyrazine-2,3-dicarboxamides are inhibitory *in vitro* but have not been evaluated *in vivo* (289, 290). Based on animal studies, interferon or immunoglobulin are not useful after onset of disease. Liver transplantation has not been attempted.

Kyasanur Forest Disease and Alkhumra Hemorrhagic Fever Viruses

Kyasanur Forest disease virus (KFDV), first isolated in Karnataka (then Mysore) State, India, in 1957 is a member of the TBE virus antigenic complex that has been subsequently termed the mammalian tick-borne virus group. The virus occurs principally in a localized region of western India, but a closely related virus, Alkhumra, has been discovered in Saudi Arabia (291). Subsequent studies have shown that Alkhumra virus is a genetic variant of Kyasanur Forest disease virus. In 2009, Nanjianyin virus was isolated from an acute febrile case in the Yunnan province of China. Nucleotide sequencing revealed the virus was nearly identical in sequence to the prototype strain of KFDV, suggesting potential laboratory contamination (292). However, the same study included a seroepidemiologic investigation of Yunnan Province from 1987 to 1990 indicating others had been infected with the virus.

Thousands of human cases of Kyasanur Forest disease have occurred in India, principally among persons working in the forest in Karnataka State. Several hundred cases occur annually, with higher rates during epidemic years. In 1983, 1,550 cases, including 150 deaths, occurred. The case-fatality rate is 1 to 2%. The transmission cycle involves ixodid ticks (*Haemaphysalis spinigera*) and wild rodents and insectivores. Wild and experimentally infected monkeys become infected and succumb to the disease. Domestic livestock are important as hosts sustaining tick populations, but their role in transmission is uncertain.

The clinical illness in humans is characterized by fever, headache, myalgia, cough, bradycardia, dehydration, gastrointestinal symptoms, leukopenia, and hemorrhagic manifestations with hypotension and shock (293). In some patients, a syndrome resembling Central European encephalitis occurs, with a febrile illness lasting a week or more, followed by remission, and then reappearance of fever and signs of meningoencephalitis. The cause of hemorrhage is unknown, but disseminated intravascular coagulation is suspected. Diagnosis is by virus isolation from blood or serology. Virus is recoverable from blood during the first week of illness (and occasionally longer). A formalin-inactivated vaccine produced in chick embryo fibroblasts is used in affected regions of India.

Alkhurma virus (ALKV) was first isolated in Jeddah, Saudi Arabia, in the 1990s from the blood of a butcher admitted to the hospital with a severe infectious syndrome. At least 256 cases have been recorded in the last 10 years; nearly all were in Saudi Arabia and involved infection via contaminated goats, sheep, or camels. The tick vectors are *Ornithodoros* spp. and *Hyalomma dromedarii*. In 2010 cases were reported in Egypt near the Egypt-Sudan border. Clinical manifestations include fever, headache, retro-orbital pain, joint pain, generalized muscle pain, anorexia, and vomiting associated with leukopenia, thrombocytopenia, and elevated levels of liver enzymes (294). Some patients have had clinical symptoms of hemorrhagic fever or encephalitis, and this has resulted in the virus sometimes being termed Alkhurma hemorrhagic fever virus. Overall, the case-fatality rate is 1 to 2%. In immunocompetent BALB/c mice, KFDV causes a neurotropic disease, whereas ALKV does not cause clinical signs of disease nor does it invade the brain (295), while, in CBA, C57BL/6J, and A/J mice, both viruses caused clinical signs of disease, but KFDV was much more virulent than ALKV (296). Overall, KFDV appears more virulent than ALKV in mice.

Treatment is supportive, and no vaccines or specific antivirals are currently available.

Omsk Hemorrhagic Fever Virus

Omsk hemorrhagic fever virus was originally isolated in 1947 from a patient with hemorrhagic fever in Siberia. The virus is a member of the TBE virus complex with a known distribution restricted to western Siberia. Approximately 1,500 cases were reported between 1945 and 1958, and the incidence in the late 1940s was high (500 to 1,400 per 100,000 population). Cases continue to occur but at considerably lower incidence. The transmission cycle involves ixodid ticks (principally *Dermacentor reticulatus*) and rodents, especially water voles (*Arvicola terrestris*). Muskrats develop epizootic illness. Sporadic cases acquired by tick bite occur in spring and summer. Muskrat hunters may become infected by contact with blood and tissues of infected animals and such cases can occur during the winter months. The disease in humans closely resembles Kyasanur Forest disease except that sequelae (hearing loss, hair loss, neuropsychiatric complaints) are relatively frequent. The case-fatality rate is 0.5 to 3%. Laboratory diagnosis is by virus isolation from blood or by serology. No specific Omsk hemorrhagic fever vaccine has been developed, but TBE vaccines apparently provide cross-protective immunity and have been used in high-risk population groups.

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Hepatitis C Virus

CHRISTOPHER KOH, QISHENG LI, AND JAKE LIANG

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Hepatitis C virus (HCV), a member of the genus *Hepacivirus* in the *Flaviviridae* family (1), is a single-stranded RNA virus that infects humans and other higher primates, and has a selective tropism to the liver. Following exposure, HCV is able to evade the host's immune system and establish a chronic, often asymptomatic, infection that may lead to liver failure, hepatocellular carcinoma, and death. Transmitted primarily by exposure to infected blood, but also through sexual and perinatal routes, the virus is estimated to infect 2.8% of the world's population (2). Originally termed non-A, non-B hepatitis, infection with HCV was a frequent cause of transfusion-related hepatitis until discovery of the virus in 1989 (3, 4) and the subsequent development of effective screening methods. Many substantial advances have recently been made in treating HCV infection, and it is now possible to cure over 90% of patients with HCV infection. These advances also provide promising opportunities for future public health efforts to effectively reduce the disease burden of this global infection.

VIROLOGY

Classification

Genotypes

Shortly after the identification of HCV, it became apparent that the virus is highly heterogeneous and that widely divergent genetic strains can be identified (5). The driving force for this variability is inherent to the replication strategy of the virus, which is mediated by an error-prone RNA-dependent RNA polymerase with a mutation rate of 10^{-4} to 10^{-5} per nucleotide (6), and a high replication level with an estimated production rate of 10^{12} virions per day (7). This mutagenic propensity is balanced by viral and host constraints. The genetic variability is manifest as genotypic, sub-genotypic, or even quasi-species differences within the same host (8). These different levels of variability probably reflect different sources and selection pressures for heterogeneity. For instance, the hypervariable region 1 (HVR-1) in the E2 gene has remarkable heterogeneity among different isolates, even within patients, probably as a consequence of its antigenicity and selective pressure by the host immune system. Genotypic variability also helps shed light on the evolutionary origin of the virus and its shared history with humans. A suggested model, based on the genotype and subtype distribution, has been described for persistent, long-term endemic foci of infection in sub-Saharan Africa and South-East Asia. This infection then rapidly spread in the last century to the western world and other countries with the advent of medical practices (blood transfusion, use of syringes) and the epidemic of injection drug abuse (9).

On the basis of phylogenetic analysis, HCV can be classified into six major genotypes, possessing sequence divergence of 30 to 35% across the viral genome (Figure 1) (10). Most genotypes can be further subdivided into subtypes, with a genomic difference of 20 to 25% (9). The viral genotypes differ with respect to geographic origin and global distribution (Figure 2) and respond differently to HCV treatment regimens (11). In general, the question of whether different genotypes are associated with disease severity remains unresolved, although genotype 3 is frequently associated with hepatic steatosis and rapid disease progression (12).

Recently, a number of human viruses have been identified that are genetically related to HCV. These include human pegivirus (HPgV) and a growing spectrum of related viruses now know to infect horses, rodents, bats, and monkeys (13). Of note, virome analysis of blood transfusion recipients has uncovered a novel human virus, human hepegivirus 1 (HHpgV-1), that shares genomic features with HCV and HPgV (14). The identification of HHpgV-1 may result in a reevaluation of the original criteria by which the genera *Hepacivirus* and *Pegivirus* are defined, and expand our knowledge of the spectrum of genome configurations of these viruses (14).

Serotypes and Antigenicity

Serotyping of HCV, although not commonly used in clinical practice, is possible. The non-structural protein NS4 has an antigenic region that is highly variable among different viral types. This variability confers type-specific antibodies in infected patients. A commercial serotyping kit, based on detection of antibodies with NS4-derived synthetic peptides, has a reported sensitivity of 77% and specificity of 94% (15). The low sensitivity, combined with the availability of more accurate nucleic acid-based methods, renders this assay less practical. In most clinical settings in the developed world, genotyping assays are preferred, although the serotyping method may be more practical for developing countries.

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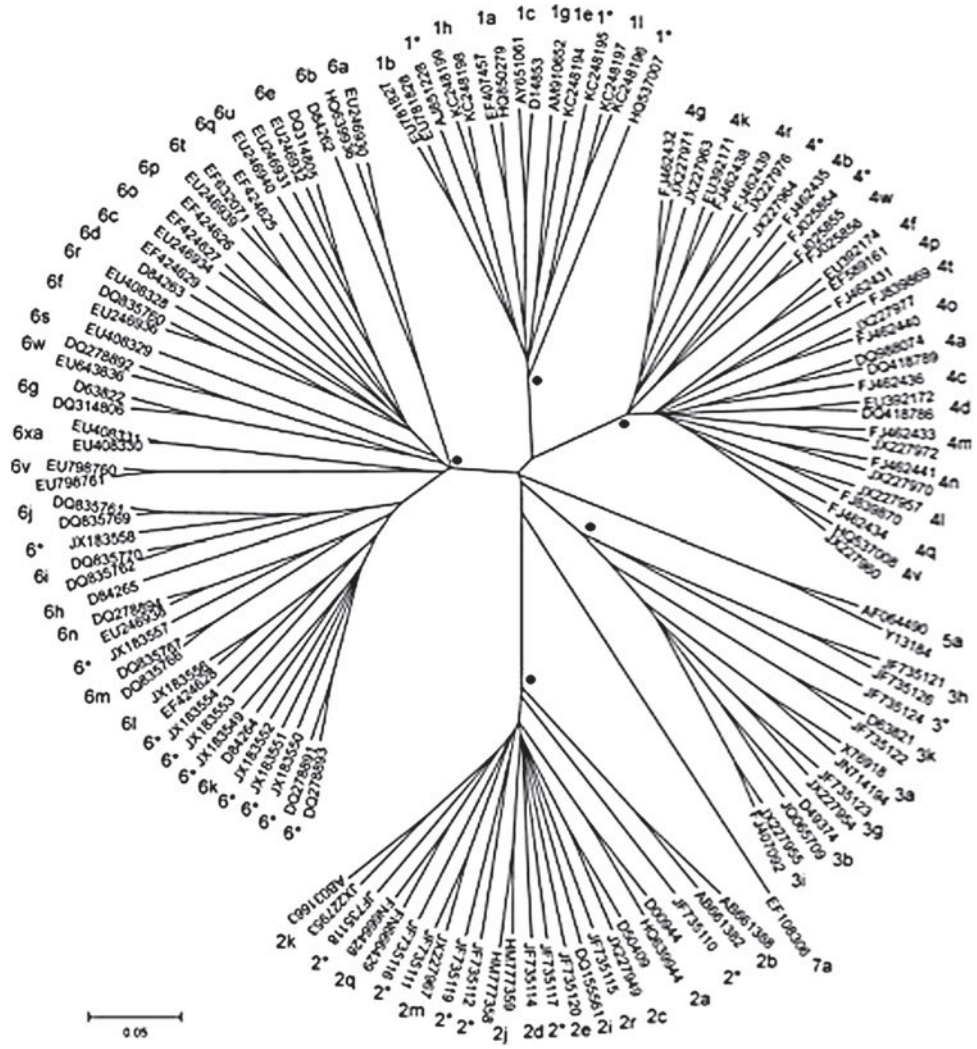

FIGURE 1 Phylogenetic tree of 129 representative complete coding region sequences demonstrating clustering to 7 genotypes and 67 subtypes. For genotypes 1, 2, 3, 4, and 6, the lowest common branch shared by all subtypes and supported by 100% of bootstrap replicates (n = 1,000) is indicated by ·. (Reprinted from Smith et al. (10) with permission.)

Composition of the Virus

Virion Morphology, Structure and Size

Despite recent progress in developing an efficient HCV *in vitro* cell culture system that enables robust production of infectious viral particles, the biochemical and morphological features of the HCV virion remain elusive. However, even before the virus was successfully identified, inactivation by chloroform was demonstrated, suggesting a lipid-membrane envelope (16) and filtration studies estimated the particle size at 30 to 60 nm. Electron microscopy studies identified HCV particles with sizes of 55 to 65 nm (17) (Figure 3a). The reported density for virus particles has a wide range of < 1.06 to 1.3 g/ml (18).

Although the HCV virion itself is difficult to visualize, by inference from other *Flaviviridae*, the putative particle is composed of a nucleocapsid composed of the core protein and viral RNA, surrounded by a phospholipid membrane in which the viral envelope proteins are embedded. Apart from lipid-enveloped virions, naked nucleocapsids may also be circulating in the plasma, perhaps playing a role in the viral interaction with the immune system. Another major form of viral particles circulating in the plasma is the lipo-viro-particle (LVP), which is rich in triglycerides and contains viral RNA, core protein, apolipoprotein B (apoB) apoE, and apoA-I (18). Recent advances in a cell culture system provide a unique opportunity to visualize and study the three-dimensional structural features of HCV virions by cryo-EM (19) (Figure 3b) and cryoelectron tomography (cryo-ET) (20).

Inactivation by Physical and Chemical Agents

Although HCV played a major role in iatrogenic infection prior to its discovery, and can be transmitted by invasive procedures, little data have been rigorously gathered on its survival in the environment and methods for inactivation. Some researchers have focused on detection (or the lack thereof) of viral RNA as a surrogate for infectivity (21) while others have examined the effect of various agents on binding of HCV to the Vero cell line (22). Many studies, however, used the related *Pestivirus* bovine viral diarrhea virus (BVDV) as a surrogate. Effectiveness of some agents is inferred from their effect on other lipid-enveloped viruses.

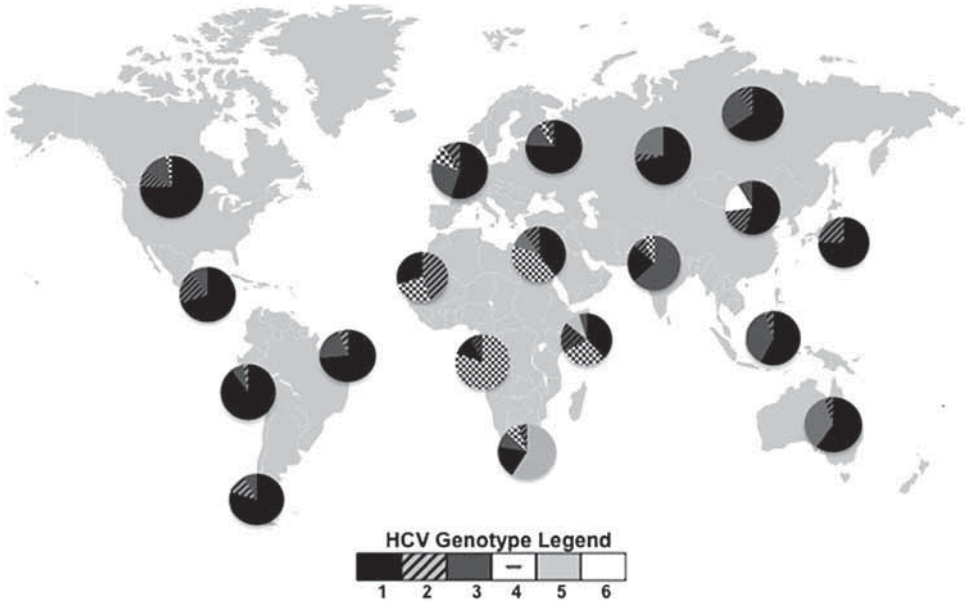

FIGURE 2 Geographic distribution of HCV genotypes. Genotype 1 is the most prevalent and can be seen worldwide. Genotype 3 is more common in South- and South-East Asia, genotype 4 is seen almost exclusively in patients from Central Africa, Egypt and Saudi Arabia, genotype 5 is mostly confined to South Africa, and genotype 6 to South-East Asia.

HCV is stable in stored serum and plasma samples at 4°C for at least 7 days (23). Even before the identification of HCV, treatment with heat, beta-propionolactone, UV radiation, or chorloform, were all shown to prevent transmission of hepatitis to chimpanzees. Treatment of blood products by solvent-detergents, photochemical methods, or ultrafiltration seems to be sufficient to prevent transmission. The current recommendations for disinfecting reusable endoscopic equipment by mechanical washing with detergent and soaking in glutaraldehyde 2% were shown to be sufficient to eliminate contamination with HCV (24). Alternative methods for cleaning medical instruments, such as the use of acidic electrolyte water or hydrogen peroxide, are also effective. The effectiveness of alcohol and diluted bleach were not formally tested against HCV, but the virus is most likely susceptible to these agents as well (25).

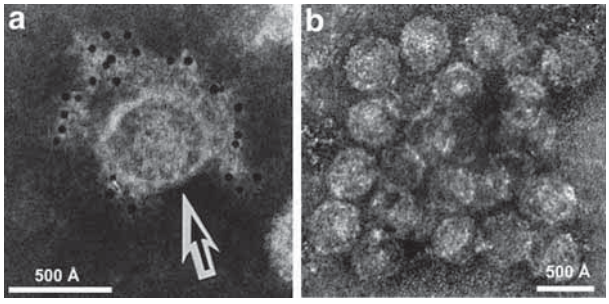

FIGURE 3 Electron microscopy of viral particles. (a) Immunogold electron microscopy of a viral particle from patient serum. The sample was incubated with Anti E1 polyclonal antibody and a secondary antibody conjugated to colloidal gold particles. An inner core (arrow) seems to be included within the particle. Reprinted from Kaito et al. (17) with permission. (b) Negative stain electron microscopy of HCV harvested from Huh-7.5 cells infected *in vitro* with HCV strain JFH-1. Spherical particles of uniform size with inner cores can be seen.

Genome Organization and Composition

The HCV genome, like that of other members of the *Flaviviridae* family, is a single-strand, positive-sense RNA of approximately 9,600 nucleotides. The genome contains a single, large, open reading frame, coding for a polyprotein of 3,300 amino acids, flanked by 2 highly conserved, untranslated regions at the 5′ and 3′ ends (Figure 4a).

The 5′ UTR (Figure 4b) contains an internal ribosomal entry site (IRES), interacting with the ribosomal machinery to facilitate cap-independent viral protein synthesis (26). The IRES has a conserved secondary structure comprising 3 domains that extend to the N-terminal core coding sequence and are essential for its function. Also contained in this region, upstream of the IRES, is a short sequence, highly conserved among all genotypes, that is recognized by a liver-specific microRNA (miRNA), miR-122 (27). Mutations introduced into this conserved segment significantly reduce viral replication *in vitro*, suggesting this endogenous miRNA enhances viral replication efficiency.

The 3′ UTR of the HCV genome is required for replication. This region consists of three distinct domains; a short variable region, a poly(U/C) tract of heterogeneous length, and a highly-conserved, 98 nt-long "X" tail organized into three stem-loop structures (Figure 4c). All of these domains are essential for efficient replication (28, 29). The second (SL II) stem-loop of the X segment is able to base pair with another loop (SL V) in the NS5B coding region, with the poly (U/C) tract probably serving as a flexible linker. As the SL V may be the binding site for the viral RNA polymerase, this closed-loop formation serves to bring the 3′ end of the positive-strand RNA into alignment with this site, to facilitate initiation of negative-strand synthesis.

Structural and Nonstructural Proteins

Translation of the HCV open reading frame produces a single large polyprotein of about 3,300 amino acids. Host viral proteases then cleave this polyprotein to produce three

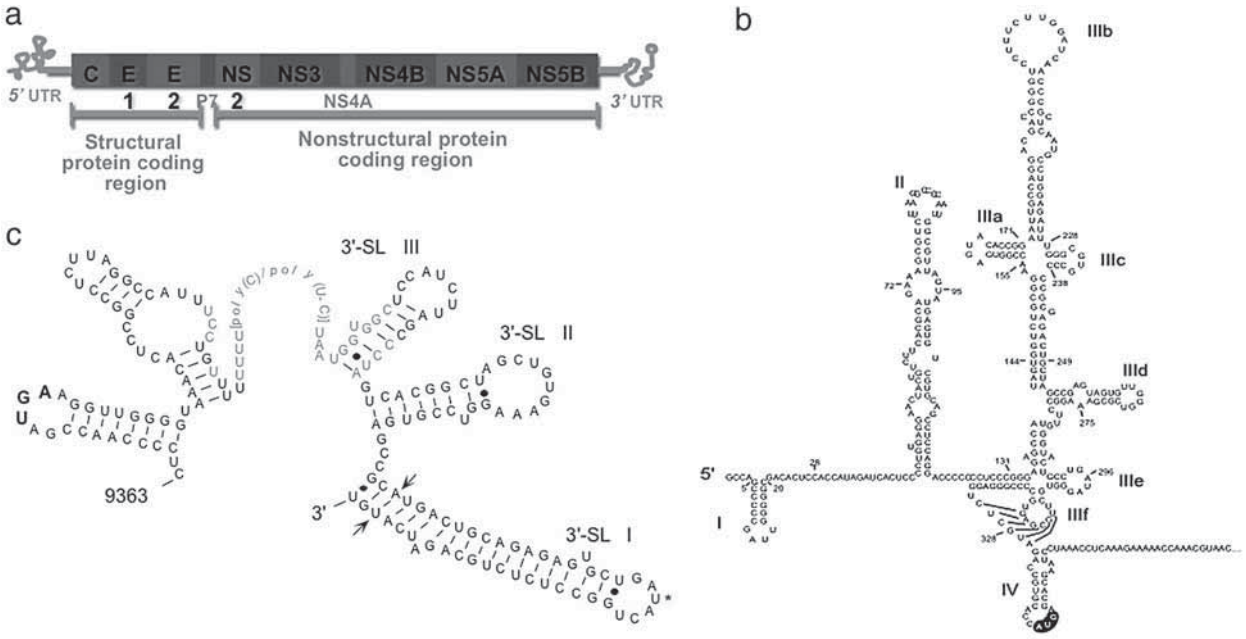

FIGURE 4 Genome structure of HCV. (a) The positive-sense single-strand RNA genome of HCV has one long open reading frame, containing genes for 3 structural and 7 nonstructural proteins, and flanked by two untranslated regions. (b) Sequence and secondary structure of the 5′ UTR. The AUG start-codon for the open reading frame is highlighted in stem-loop IV. (c) Sequence and secondary structure of the 3′ UTR region. Arrows indicate variable base pairs in the stem of SL1 and the asterisk denotes the variable nucleotide in the loop of SL1. UTR—untranslated region.

structural (core, E1, E2) and seven nonstructural proteins (p7, NS2—NS5) (Table 1).

The core protein is a basic, RNA-binding protein that together with the viral genome forms the viral nucleocapsid (30). After translation and cleavage of the first 191 amino acids of the viral polyprotein, it undergoes a series of post-translational modifications, before it becomes functional. Core is able to interact with multiple host proteins and induce host gene expression, being implicated in the development of liver steatosis, carcinogenesis (31), apoptosis, and immune modulation. The association of core with the lipid droplet (LD) and interactions with NS5A are essential for production of infectious viral particles (32, 33). Core protein also recruits viral replication complexes (RCs) to LD-associated membranes and induces the accumulation of LDs in hepatocytes to enhance viral assembly.

Translation from an alternative reading frame of the core encoding region, with a +1 codon frame shift, yields a protein called F-protein or ARFP (34). The reading frame for this protein does not start with the canonical AUG start codon, and translation is probably initiated by a programmed ribosomal frame-shift. The evolutionary conservation of this frame-shift product, and the presence of antibodies to this protein in the sera of infected patients suggest that this protein is produced *in vivo* and probably has a role, yet to be elucidated, in the viral replication cycle and pathogenesis.

The two envelope proteins, E1 and E2, are type I transmembrane glycoproteins with an N-terminal ectodomain. These proteins are associated with the ER membrane after cleavage, where their ectodomain undergoes modification by N-linked glycosylation (35). They form non-covalent heterodimers that function as the building blocks for the assembly of the virion. Although both proteins facilitate cell entry, E2 is probably the major viral protein to interact with host cell-surface molecules and is thought to mediate viral entry (36). Several highly variable regions (HVRs) have been identified in E2, differing by up to 80% among HCV genotypes. While HVR1 represents an immunodominant region that elicits type-specific neutralizing antibodies, HVR2 may modulate receptor E2 binding and play a structural role in glycoprotein assembly and virion infectivity (37). E1 protein contains a putative fusogenic sequence that may be involved in viral fusion (38).

P7 is a hydrophobic 63 amino acid protein that is associated with endoplasmic reticulum and mitochondrial associated membranes. Considered as a class IIA viroporin, HCV p7 is able to form a multimeric cation channel that can be blocked by amantadine (39). p7 is dispensable for HCV replication *in vitro*, but is essential for the formation and release of infectious HCV particles through its ion channel and pore-like activity (40). It is possible that the pore formed by p7 modulates intra-ER pH to protect viral glycoproteins during maturation and export. Besides its channel activity, p7 also acts in concert with core, envelope proteins, and NS2 to facilitate HCV assembly (41, 42). Several viroporin inhibitors that antagonize p7 ion channel activity are currently under clinical investigation for their anti-HCV effects (43).

The nonstructural 2 (NS2) protein encodes a cysteine protease whose function is stimulated by cofactor domains in NS3 (44). The protease has an autocatalytic activity, cleaving the polyprotein precursor at the NS2/NS3 junction to free, fully functional NS3, and thus promote viral RNA replication. NS2 also acts as a central organizer of HCV assembly that is independent of its protease activity, but may exploit a complex network of interactions with the envelope and various other nonstructural proteins (45, 46).

The next nonstructural protein, NS3, has a chemotrypsin-like serine protease in the N-terminus (47) followed by an NTPase/RNA helicase in the C-terminus (48). The

TABLE 1 Viral proteins and their function in the viral replication cycle

Name	Size (amino acids)	Function	Intracellular location	Comment
Core	171	Viral nucleocapsid	ER membranes, membranous webs, lipid droplets	Associates with viral replication complexes (RCs) and LDs for HCV assembly
E1	192	Viral envelope	RER lumen (membrane anchored)	Glycoprotein; Heterodimer formation with E1; Possibly involved in fusion
E2	363	Viral envelope	RER lumen (membrane anchored)	Glycoprotein; Heterodimer formation with E2; Binds to CD81 and other entry factors
p7	63	Cation channel, viroporin	RER membrane, mitochondrial membranes	Inhibited by amantadine & amiloride
NS2	217	Cysteine protease and viral assembly	RER membrane	Protease activity when joined with NS3
NS3	631	N-terminal serine protease, C-terminal RNA helicase	Cytosol (anchored to ER membrane by NS4A)	Protease activity dependent on NS4A co-factor; Cleaves RIG-I adaptor MAVS and mediates viral evasion to innate immunity
NS4A	54	Protease cofactor	ER membrane	HCV RNA replication and virion assembly
NS4B	261	Scaffold for replication complex	ER membrane	Induces the formation of "membranous webs"
NS5A	447	Regulating replication and assembly	ER membrane	Phosphoprotein; RNA-binding
NS5B	591	RNA-dependent RNA polymerase	ER membrane	
F/ARFP		Unknown	Unknown	Product of an alternate reading frame of the core-encoding region

serine protease activity requires NS4A as a cofactor, which anchors the complex to the ER membrane and changes the catalytic site conformation through its N-terminal domain (49). The C-terminal portion of NS4A interacts with other replicase components and contributes to HCV RNA replication and virion assembly (50, 51). The NS3 NTPase/RNA helicase acts as a molecular motor, unwinding double-stranded RNA in an ATP-dependent manner and facilitating the activity of NS5B polymerase. It is required for RNA replication and meanwhile plays a role in viral assembly (52). Interestingly, NS3/4A also targets and cleaves several host cellular proteins, including the RIG-I adaptor MAVS and the TLR3 adaptor TRIF, supporting its role in mediating viral evasion to immune response and HCV persistence (53, 54). The NS3 protease is an attractive target for developing antiviral therapy, and several specific NS3/4A protease inhibitors are approved or being investigated as direct antiviral agents (DAAs) (see below).

The role and structure of NS4B are relatively less known. This hydrophobic 27 kDa protein is an integral membrane protein across the ER membrane, and incudes the formation of membranous webs, unique membranous structures that serve as a scaffold for HCV replication complexes. NS4B also interacts with other viral nonstructural proteins, binds to viral RNA, and contains a GTP binding motif that is important for viral replication (55). Sequence variations in the protein have a significant effect on the efficiency of viral replication (56). In addition, NS4B harbors NTPase activity and has been shown to play a role in viral assembly (57).

The 447 amino acid nonstructural protein NS5A is a hydrophilic, proline-rich protein, anchored to the ER membranes by its C-terminus (58). NS5A plays an important role in both HCV RNA replication and viral assembly (59). The protein is extensively phosphorylated by host kinases, such as the α isoform of casein kinase I (CKIα) (60) and casein kinase II (CKII) (61), and the degree of its phosphorylation is inversely related to the efficiency of viral replication *in vitro*. Adaptive mutations that reduce the degree of hyperphosphorylation are required for efficient replication of HCV replicons in tissue culture; however, the same mutations inhibit infectivity *in vivo*. This inverse relationship between *in vivo* infectivity and *in vitro* replication suggests that NS5A may act as a molecular switch, directing replication or assembly of virions according to the degree of its phosphorylation. Mutations in the interferon sensitivity-determining region (ISDR) within NS5A were initially thought to play a major role in the resistance to interferon of genotype 1 strains, but this was subsequently questioned by other studies (62). NS5A interacts extensively with viral elements and cellular proteins (63). Like core and NS3/4A, NS5A is probably important for the ability of HCV to evade the host's antiviral immune response.

The last protein encoded in the viral polyprotein is NS5B, the RNA-dependent RNA polymerase (64). This 68 kDa enzyme has the ability to initiate RNA synthesis *de novo*. The crystal structure of the catalytic domain demonstrates palm, finger, and thumb subdomains similar to other polymerases, including the conserved GDD sequence. A transmembrane C-terminal domain anchors the catalytic site to the membranous webs discussed below. This protein is responsible for the synthesis of the negative-strand intermediate and then the positive-strand viral genome. This essential role, and the well-defined catalytic activity, makes this enzyme another attractive target for the development of specific HCV inhibitors (see below).

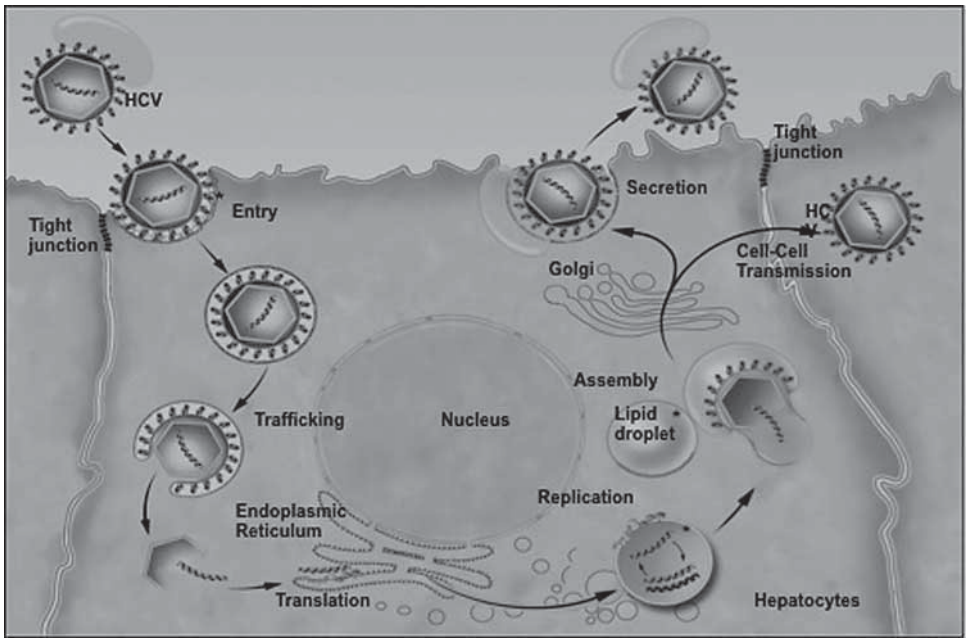

FIGURE 5 Viral replication cycle. Binding of the virus to the cell surface and internalization are followed by release of the viral RNA, harnessing of the cellular ribosomes, and protein translation and processing. The viral proteins, associated with the ER membrane, promote the formation of replication complexes anchored to lipid membranous webs in which the RNA dependent polymerase NS5B creates copies of the viral RNA through a negative strand RNA intermediate. HCV RNA is then packaged into membrane-covered viral particles and exported from the cell, presumably through the exocytosis pathway.

Biology

HCV Replication Cycle and Host Dependencies

The replication cycle of HCV is not fully understood, although great advances have been made since the discovery of the virus. The replication cycle can be divided into multiple stages: viral binding and entry to the cell, post-entry trafficking of viral genome, polyprotein translation and processing, RNA replication, packaging and assembly of the virion, and release or secretion (Figure 5). Each of these steps interacts with, and depends largely on, cellular pathways and host factors (65). Identification of these host dependencies may provide not only potential antiviral targets, but also critical insights into mechanisms of HCV-mediated pathogenesis and chronic liver disease.

Binding and Entry

Great advances have been achieved in elucidating HCV entry pathway, mechanism, and cellular factors involved in the entry processes. Entry steps play central roles in cell tropism and species specificity. The highly coordinated HCV entry process exploits multiple cellular molecules, including tetraspanin CD81 (66), the tight junction proteins, claudin-1 (CLDN1) (67) and occluding (OCLN) (68), the receptor tyrosine kinases epidermal growth factor receptor (EGFR), and ephrin receptor A2 (EphA2) (69), Niemann-Pick C1-like 1 (NPC1L1) (70), and transferrin receptor 1 (70). The presence of both E1 and E2 in a non-covalent heterodimer formation is essential for viral entry (71). Several cell-surface molecules, mostly identified by screening for the ability to bind a soluble E2 protein, have been suggested as putative receptors that could facilitate virus-cell interaction and cell entry. The process is likely to involve several of these molecules, sequentially or in unison, as none alone was shown to be sufficient for entry. The 25 kDa tetraspanin CD81, a ubiquitous surface protein, is able to bind E2 (66) and is required for cell entry in a density-dependent manner. E2-CD81 binding sites have been shown to be major targets for broadly neutralizing antibodies, which thereby efficiently inhibit viral binding and entry (72, 73). There is, however, evidence to suggest that CD81 is not required for binding but acts at a post-binding stage.

Another putative viral receptor is scavenger receptor class B type I (SR-BI), an 82 kd, E2-binding protein (74), expressed on hepatocytes, dendritic cells, and steroidogenic tissue (adrenals and ovaries), and involved in binding of HDL, VLDL, and oxidized LDL. SR-BI plays multi-stepwise roles during HCV entry. The initial binding of HCV to SR-BI is mediated by HCV-associated lipoproteins, such as apoE, present on LVPs. The lipid uptake activity of SR-BI may assist the exposure of CD81 binding sites on E2 and transfer of viral particles to CD81. The effect of SR-BI on HCV entry is enhanced by HDL cholesterol and inhibited by oxidized LDL. In addition, SR-BI-E2 interaction enhances HCV infection at a post-binding stage. Indeed, HCV entry employs a complex interplay between lipoproteins, SR-BI, CD81, and HCV envelope glycoproteins.

A few other molecules are involved in this process. Highly sulfated heparin sulfate, a liver-specific form of heparin sulfate, was shown to bind E2 (75), and it may be the initial binding surface molecule. DC-SIGN and L-SIGN, two members of the C-type lectin family, were also shown to bind E2 and HCV particles. However, these proteins are expressed by dendritic cells and liver sinusoidal endothelial cells, respectively, but not by hepatocytes. Thus, they probably promote infection *in trans* by capturing virions and transporting them to the vicinity of hepatocytes (76). The LDL receptor (LDLR) is also involved with the internali-

zation of HCV (77), probably through association of viral particles with lipoproteins, because entry of naked HCV pseudoparticles is not mediated through this receptor (71).

CLDN1, a component of tight junctions that is highly expressed in the liver, was identified as an essential HCV entry factor (67). HCV E1 but not E2 may directly interact with CLDN1 (78). CLDN1 contributes to the post-binding steps of HCV entry by interacting with CD81, forming a complex critical for virus internalization (67, 79). OCLN, another tight junction protein, functions at a post-attachment step during HCV entry (68). Together with CD81, OCLN defines the species tropism of HCV entry (80, 81).

Receptor tyrosine kinases EGFR and EphA2 are also important HCV entry factors (69). EGFR-dependent signaling promotes CD81-CLDN1 association, thus facilitating HCV entry. The cholesterol transporter Niemann-Pick C1-like 1 (NPC1L1) and the iron uptake receptor, transferrin receptor 1 (TfR1), were found to be additional entry factors (70, 82). The precise functions and mechanisms of these cellular factors remain to be addressed.

Entry into the cell is clathrin and pH dependent, suggesting that after binding, the virus is endocytosed and that membrane fusion occurs in the endosome. In summary, although several cell surface molecules have been shown to bind viral proteins or particles and to facilitate cell entry, the process *in vivo* most likely involves multiple molecules, interacting simultaneously or in sequence to bind, attach, endocytose, and internalize the virus.

Protein Translation and Processing

Following internalization and release of the viral RNA from the capsid, protein translation ensues. The IRES in the 5′ UTR is able to harness the 40S ribosomal subunit in the absence of other initiation factors (needed for cap-dependent translation) and direct it to the AUG start codon of the viral polyprotein. The eIF2-Met-tRNA-GTP ternary complex, eIF3, and the 60S subunit are then recruited to form the active 80S ribosome (26).

Following translation, the large polyprotein product is co- and post-translationally cleaved by host (signalase and signal peptide peptidase) and viral (NS2/3 and NS3/4A) proteases to release ten mature HCV proteins (83). Four signal peptides are located in the polyprotein sequence: at the core protein and E1 junction, between E1 and E2, E2 and p7, and at the p7 and NS2 junction. These signal peptides direct the elongating protein to the endoplasmic reticulum membrane and determine the location of the future cleavage products: cytosolic, ER-membrane associated, or intraluminal. Cleavage at the site of the signal peptides is by the ER signal peptidase (84). The NS2/3 junction then undergoes autocatalytic cleavage following dimerization (85). Further downstream processing is dependent upon the NS3/4A protease, which cleaves the NS3/4A, NS4A/4B, NS4B/NS5A, and NS5A/NS5B junctions, with NS4A as a required cofactor (47).

Less is known about cellular proteins involved in HCV IRES-mediated translation. Recent integrative functional genomics studies identified PIAS1, USP11, and several other cellular genes as putative host factors that promote HCV protein translation (65).

RNA Replication

RNA replication occurs in a membrane-anchored complex of the viral nonstructural proteins and RNA, termed the replication complex or the replicase. Replication *in vitro*, and perhaps *in vivo* as well, takes place on unique membranous webs, thought to be derived from the ER membranes by the action of NS4B (86).

HCV replication relies on miR-122 (27), a liver-specific host microRNA that recruits Argonaute 2 to the 5′ end of the viral genome (87), stimulating viral RNA synthesis, and protecting it from degradation by exonuclease Xrn1 (88). Antagonism of miR-122 with an antisense oligonucleotide results in long-lasting suppression of HCV viremia in chimpanzees and humans, and hence constitutes a novel therapeutic strategy against chronic HCV infection (89, 90).

Taking advantage of RNAi-based screening and mass spectrometry interactome approaches, a large number of HCV host dependencies influencing translation and replication have been identified (91–93). These include cyclophilin A (CypA), a peptidyl-prolyl cis-trans isomerase, and phosphatidylinositol-4-kinase-III-α (PI4KIIIα), a kinase and central player in HCV replication. CypA inhibitors, such as cyclosporine A and nonimmunosuppressive analogs alisporivir (DEBIO-025) (94) and SCY-635 (95), exhibit potent anti-HCV activity and are being developed as host-targeting antivirals (HTAs).

Assembly and Release

The process of packaging and export of HCV virions is assumed to involve budding of virions into the ER membrane and export through the secretory pathway, but detailed mechanisms involved in HCV assembly and release are yet to be delineated, although an intricate interaction with host cell lipid metabolism has been suggested (96, 97). In the early step of assembly, the core protein recruits nonstructural proteins and viral replication complexes to lipid droplet (LD)-associated membranes, but the subsequent steps remain unclear (32, 98). Multiple host factors have been identified to participate in HCV assembly. Most notable is ApoE, which appears to be incorporated into infectious HCV particles through interaction with NS5A (99, 100). Other lipid metabolism genes are also required for HCV assembly, for example MTTP, a microsomal triglyceride transfer protein that is essential for VLDL synthesis (101), and DGAT1, a lipogenic enzyme that specifically interacts with and translocates HCV core to the viral assembly sites (102). Recently, the IκB kinase-α (IKK-α) has also been shown to be a critical host factor mediating HCV assembly (103). HCV, through its 3′ UTR, interacts with DEAD box polypeptide 3, X-linked (DDX3X) to activate IKK-α, which translocates to the nucleus and induces a CBP/p300-mediated transcriptional program involving sterol regulatory element-binding proteins (SREBPs). This innate pathway induces lipogenic genes and enhances core-associated lipid droplet formation to facilitate viral assembly (103).

The host dependencies for HCV secretion are largely unknown. The current model suggests a role of VLDL secretory pathway and trans-Golgi network (TGN)-recycling endosomes in the release of HCV particles (101, 104, 105).

Cell Culture and *In Vitro* Model Systems

Since the discovery of HCV, the lack of a robust cell culture system capable of supporting efficient productive infection of HCV had been a major obstacle to the study of this pathogen and the development of countermeasures. Surrogate model systems, such as production of HCV-like particles and HCV retro-pseudoparticles, provide valuable tools to study virus-cell interactions (106), but none of these *in vitro* model systems produce infectious viral particles. The development of replicon systems, instrumental in creating a

TABLE 2 Virologic model systems

Model system	Method	Proteins expressed	Life cycle stages studied	Infectivity *in vivo*	Comments
HCV-like particles (HCV-LP)	Assembly of envelope proteins in insect and mammalian cells	E1, E2	Binding and entry	-	
HCV pseudotype particles (HCVpp)	Envelope proteins assembled to a retroviral or lentiviral core particle	E1, E2	Binding and entry	-	
Subgenomic replicon	Expression driven by EMCV IRES	NS2-NS5B	Replication	-	Requires culture-adaptive mutations
Full genomic replicon	Expression driven by EMCV IRES	Entire genome	Replication	-	Requires culture-adaptive mutations
JFH-1 infectious strain (HCVcc)	A genotype 2a strain from a case of fulminant hepatitis	Entire genome	All	+++	Chimeras with other strains developed
DNA-ribozyme expression system	Transfection of viral cDNA between 2 ribozymes	Entire genome	All	++	Not sequence specific

system to study viral replication, consist of a bicistronic construct containing the non-structural genome region (NS3-NS5B) downstream of the encephalomyocarditis virus (EMCV) IRES and a selectable marker driven by the HCV IRES (Table 2) (107). Transfection of these subgenomic replicons to human hepatoma Huh7 cells resulted in viral replication. However, efficient viral replication depends on the appearance of adaptive mutations, especially in NS5A (108). This system is naturally limited by the absence of the structural proteins and hence is not amenable to studying the packaging and export process. Further modifications of the replicon system included the production of monocistronic subgenomic replicons and of a full-length replicon. To date, subgenomic replicons have been constructed for six major HCV genotypes, and have greatly contributed to the discovery of DAAs that are currently licensed or under development. A major breakthrough for investigating the process of HCV entry was the development of HCV pseudoparticles (HCVpp)—defective retroviral particles expressing HCV envelope glycoproteins on their surface (71, 109). Using HCVpp system, a variety of viral entry factors and molecular pathways have been uncovered.

Wakita et al. (110) transfected hepatoma cells with a full-length cDNA cloned from a patient with fulminant hepatitis C. This strain, termed JFH-1 and classified to genotype 2a, replicates efficiently in tissue culture without adaptive mutations and produces viral particles that are infectious to hepatoma cells *in vitro* and to chimpanzees *in vivo* (111, 112). Studies utilizing this strain and chimeras derived from it have greatly enhanced our knowledge of the complete viral life cycle. However, the JFH-1 strain is rather unique and information garnered from studying this strain may not be applicable to other more common strains, in particular genotype 1. Similar results have been obtained with a modified genotype 1 virus, but adaptive mutations were required to maintain *in vitro* replication and infectivity (113).

These approaches are all sequence dependent, and thus results are not necessarily generalizable to other viral strains or genotypes. One sequence-independent system (114) involves a viral cDNA inserted between two self-splicing ribozymes and transfected into human hepatoma cells (114). This system can be applied to different HCV strains and genotypes to produce HCV particles capable of infecting chimpanzees (115). Others have been able to infect non-transformed human fetal hepatocytes in culture with HCV from genotypes 1, 2, 3 from patient sera and demonstrated the release of infectious virions to the medium (116). Recently, a single human cDNA, SEC14L2, enabled replication of diverse HCV genotypes in hepatoma cell lines (117). SEC14L2-expressing Huh7.5 cells also supported HCV replication directly from patient sera. Mechanistically, SEC14L2 enhances HCV infection by facilitating vitamin E-mediated protection against lipid peroxidation (117). This discovery represents an important breakthrough in developing an HCV cell culture (HCVcc) system in which the virus can be propagated without the need for adaptive mutations, thereby opening up new avenues for studying HCV biology, including drug resistant clinical variants that are emerging even in the new era of DAAs.

The HCVcc system enabled for the first time studies of the entire viral replication cycle, and also led to biophysical and ultrastructural characterization of the HCV virion. Importantly, HCVcc is infectious in chimpanzees and human liver-chimeric mice, permitting the evaluation of vaccine candidates *in vivo* and the development of genetically humanized mice (118). In addition, the recently developed chimeric HCVcc models cover a wide range of viral variants, and will potentially be of importance for future tailoring of the optimal regimens that address all HCV genotypes (119).

To date, cells most permissive to HCV infection *in vitro* are a human hepatoma cell line, Huh7, and its derivatives, including the widely used, RIG-I and innate immunity incompetent Huh7.5 cells. A limitation of the Huh7 cell lines lies in the lack of cell polarity. As such, certain aspects of the viral replication cycle and interaction with lipoproteins may not be reproduced in these cells (120). The polarized HepG2 cells, though refractory to HCV infection due to lack of CD81 expression, can be engineered fully permissive to HCV and support the entire HCV replication cycle upon ectopic expression of human CD81 and miR-122 (121). Primary human hepatocytes (PHHs) represent the natural host for HCV, and HCVcc systems based on PHHs may be

optimized to achieve reproducible and sustained infection in a physiologically relevant manner. Differentiated hepatocyte-like cells (HLCs) derived from both human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) are permissive to infection of HCVcc and HCV-positive sera *in vivo*, providing exciting new models for study of HCV biology, pathogenesis and clinical HCV isolates (122–124).

Host Range and *In Vivo* Models

HCV appears to infect only humans. A study in Gabon failed to detect hepatitis C virus infection in 316 wild-born primates of different species (125). As shown even before HCV was identified, chimpanzees can be experimentally infected with HCV. Although chimpanzees can develop a persistent infection like humans and develop similar inflammatory liver lesions, there are some important differences in disease behavior among the species. Persistent infection occurs after inoculation in only 30 to 40% of chimpanzees (as opposed to 85% in humans), and fibrosis and cirrhosis do not occur (126). The limited availability and the endangered status of chimpanzees, in addition to the financial cost, led to a search for other animal models. Tree shrews (*Tupaia belengeri chinensis*), a species distantly related to primates, can be transiently infected with HCV (127). HCV may utilize the tupaia orthologs of CD81, SR-BI, CLDN1, and OCLN to gain entry into tupaia hepatocytes (128) and induces mild hepatitis within the acute phase of infection (129).

The mechanism for the selective liver tropism is not clear but most likely involves selectivity of one or more of the cell surface molecules responsible for cell entry, although the specific molecule has not been identified. The species tropism of CD81 has been investigated. HCV E2 glycoprotein was shown to bind CD81 from tamarins (*Saguinus* sp.), which are not susceptible to HCV infection (130). Similarly, expression of CD81 from African green monkeys and rats in hepatoma cells confers susceptibility to HCV entry (131).

Mice are naturally resistant to HCV infection—both viral entry and replication are fairly limited in murine cells. Intriguingly, transient expression of human CD81 and OCLN by adenoviral gene delivery enables mouse hepatocytes to be infected by HCVcc, and the derived mouse model has been applied for evaluation of vaccine candidates and entry inhibitors (73, 80). Subsequently, transgenic expression of human HCV entry factors combining blunting of the mouse innate immune responses has allowed the recapitulation of the entire HCV replication cycle, albeit at low levels (81). However the level of HCV replication is still much lower in these reconstituted mouse cells, suggesting other unidentified tropic factors are important.

In an attempt to overcome viral selectivity and establish a small animal model, several groups developed immunodeficient mouse strains harboring chimeric human-mouse livers (132, 133). HCV can replicate in the human-derived hepatocytes of these mice, but the absence of an effective immune response limits the value of this model in studying the pathogenesis of hepatitis C.

EPIDEMIOLOGY

Geographic Distribution

Infection with HCV is a global problem affecting up to 2.8% of the world population (134). Great variability exists in seroprevalence rates among various countries and geographic regions (Figure 6) reflecting differences in public health practices, transmission patterns, and surveillance schemes (135). In the developed world, injection drug use is currently the most common mode of infection, and overall seroprevalence is generally low, ranging from 0.6% in Germany to 2.2 to 2.3% in Italy and Japan. The seroprevalence in the United States is reported at 1.6% (135). In the developing world, unsafe injections and contaminated medical instruments seem to be a major risk factor (136). Egypt has the highest reported seroprevalence rate, 22%, attributable to

FIGURE 6 World distribution of HCV seroprevalence. The highest prevalence is in Egypt. Other countries with high prevalence of infection include Mongolia, Bolivia, and several sub-Saharan nations.

mass public health campaigns of parenteral antischistosomal treatment (137). The WHO estimates that in the year 2000, 2 million people worldwide were iatrogenically infected by reused injection equipment (138).

Transmission

Routes

The major route of HCV transmission is parenteral. In the past, transfusion of blood or blood products was the most common means of acquiring infection in the developed world. Post-transfusion hepatitis rates in patients undergoing open-heart surgery, for example, were as high as 33%. Products manufactured from pooled donor blood (such as anti-D globulin or coagulation factor concentrates) were implicated in large outbreaks of infection (139). However, changes in donor selection, including elimination of donor remuneration, exclusion of high-risk populations, screening donated blood for ALT elevations, and the use of recombinant clotting factors, greatly reduced the risk of transmission even before HCV was actually identified (140) (Figure 7). With the implementation of specific HCV tests, first serologic and then nucleic acid based, the risk of transfusion-associated HCV decreased further and is now estimated at 1:600,000 per unit.

With the elimination of blood transfusion as a risk factor, most of the parenteral exposure in the developed world is the result of injection drug use (141). Practices like needle sharing, back-loading, and sharing cotton, rinse-water, and other paraphernalia are associated with transmission. The seroprevalence of HCV antibodies increases with drug use duration, reaching a rate of 60 to 85% for persons using drugs for more than 6 years. Snorting cocaine is also a risk factor for acquiring HCV, probably through contamination of shared snorting devices and nasal mucosal injury. Various risk-reduction measures, such as needle exchange programs, have shown some benefit in reducing transmission, but the overall effect is not optimal (142).

Iatrogenic transmission of hepatitis C virus has been associated with multiple medical and dental procedures (143). Cases of transmission were reported to be associated with gastrointestinal and pulmonary endoscopy, ambulatory surgical procedures, chemotherapy, administration of anesthesia, radiologic procedures, and in general any procedure involving parenteral access. Patients on hemodialysis are at an especially high risk for nosocomial infection (144) and a large number of outbreaks in dialysis units has been reported. The risk for dialysis patients can be decreased significantly by segregating patients to dedicated rooms and equipment based on HCV serology. In general, most cases of iatrogenic transmission in developed countries are associated with breaches of universal precautions and inadequate infection-control practices, especially with the use of multi-dose vials. The source for most nosocomial outbreaks is a chronically infected patient, although transmission from an infected healthcare worker has been documented in rare cases. As previously discussed, in the developing world, iatrogenic infection is associated with reuse of medical instruments, substandard hygienic practices, and treatment by non-professionals.

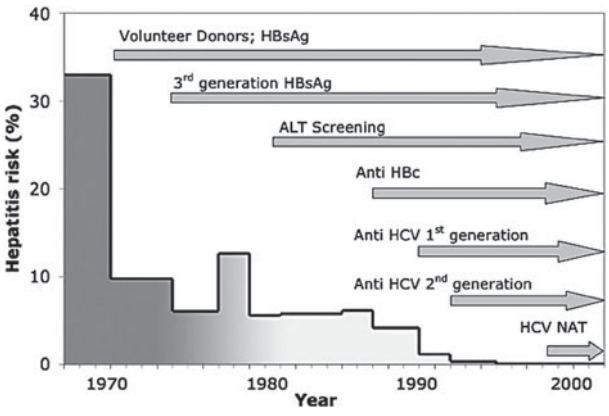

FIGURE 7 The evolution of screening methods for donated blood and the corresponding decrease in transfusion-related hepatitis. Data shown for non-A, non-B hepatitis before the availability of anti-HCV testing, and for HCV afterwards. HBsAg—hepatitis B virus surface antigen. Anti-HBc—hepatitis B virus core antibodies. NAT—nucleic acid technology. (Adapted from reference 140.)

Healthcare workers are at risk for acquiring HCV infection through needle-stick injuries. The risk is related to the size of the needle, the depth of the injury, intravenous or intra-arterial needles, higher viral load, and male sex (145). Overall, the risk of infection from a needle-stick is usually quoted at 3% based on early reports, although a later analysis suggested the risk is only 0.5% (146).

HCV can be transmitted vertically from mother to child. The risk of transmission is estimated at 2 to 5.5% (147, 148) in HCV mono-infected mothers. The major risk factor for vertical transmission is co-infection with HIV, which increases the transmission risk significantly (149). Other risk factors include maternal viral RNA and ALT level, prolonged labor (more than 6 hours after rupture of the membranes), use of internal fetal monitoring devices during labor, and female sex of the newborn. The risk of transmission with vaginal delivery is not greater than with a caesarean section, and the latter should not be recommended routinely (150). Infection can occur either *in utero* or during delivery; whether postpartum transmission also occurs is not clear. Although HCV has been detected in breast milk from infected mothers, it does not seem to be transmissible by breast feeding and this should not be avoided by HCV positive women (151).

Sexual transmission of HCV was documented in several case reports, but the actual risk for sexual transmission is hard to determine (152). Sexual exposure was found to be the only risk factor in 15% of acute hepatitis C cases (153), and hepatitis C is somewhat more common in high-risk populations for sexually transmitted diseases: HIV infected, sex workers, men who have sex with men, multiple sex partners, and presence of other sexually transmitted diseases. However, studies of long-term monogamous heterosexual spouses of HCV-infected patients demonstrated a relatively low rate of infection, and those studies that utilized detailed analysis found that most infections were associated with other possible risk factors, such as drug abuse, sharing razors, high-risk sexual practices, etc. Moreover, when sequence analysis was used, only a small number of co-infected sexual partners actually harbored the same strains. Two recent studies of low-risk monogamous couples (154, 155) found rates of HCV seropositivity of 2% to 3.8% in spouses of HCV-infected patients, although most infections were attributable to other risk factors. Prospective follow-up at up to 10 years did not identify any new case of interspousal transmission. Thus, sexual transmission is possible but appears to be very inefficient. This observation probably relates to a low titer of the virus in vaginal secretion, semen, and saliva, a lack of

target cells in the genital system, and possibly other mechanisms as well.

The efficiency of sexual transmission during acute hepatitis C is less well defined. In a study of Egyptian healthcare workers with documented acute hepatitis C (156), 15% of spouses developed viremia, which spontaneously resolved in half of them. The breadth and magnitude of the polyclonal $CD4^+$ and $CD8^+$ T-cell response against HCV was greater in patients who eventually cleared viremia when compared to those who progressed to chronic infection. Interestingly, significant T-cell responses were also seen in some spouses of infected patients, although these spouses never developed viremia or seroconverted. This finding suggests that the host immune system plays a major role in the control of sexually transmitted HCV.

Sexual transmission of HCV by men who have sex with men (MSM) has been well documented in urban areas of North America, Europe, Australia, and Asia (156, 157). In the presence of HIV infection, acute HCV infection has a higher likelihood of chronicity with a more rapid progression to cirrhosis (158). Factors associated with transmission in MSM with HIV include higher viral loads, traumatic sexual practices, the presence of other sexual transmitted infections, and decreased sexual inhibition related illicit drug use in MSM (159–163).

Risk Factors

Risk factors for infection with HCV stem directly from the routes of transmission discussed above. Thus, in the western world, those at the highest risk for newly acquired infections are individuals who use intravenous drugs and share needles. Among non-drug users, transfusion of blood prior to 1992 or of clotting factors prior to 1987 constitutes a major risk. Patients on hemodialysis, past or present, are also a major risk group. Individuals incarcerated at correctional facilities are at a high risk of exposure to hepatitis C virus, reflecting the prevalence of IV drug use and high-risk sexual behavior in this population. Estimates of HCV seroprevalence in incarcerated inmates range from 16 to 41% with chronic infection detected in 12 to 35% (164). Similarly, homeless persons with severe mental illnesses are also at a high risk for infection.

Promiscuity and high-risk sexual behavior were identified as risk factors for HCV seropositivity in cross-sectional studies (165). Although the prevalence is higher in female sex workers, it seems to be related more to drug-abuse habits than to sexual transmission (166). Similarly, although epidemics of acute hepatitis C were recorded among MSM, this is mostly related to high-risk behavior and drug use (167).

Ethnicity and sex significantly modify the risk for HCV infection. In the United States, men are at a higher risk than women with a prevalence of 2.1% and 1.1%, respectively (135) and African Americans have higher rates than Caucasian Americans. The highest rates were observed in non-Hispanic black males aged 40 to 49 years, for whom the prevalence is a striking 13.6%.

PATHOGENESIS IN HUMANS

Incubation Period

Since many acute HCV infections are asymptomatic, it is difficult to define a precise incubation period. However, the relative kinetics of viral levels, liver enzymes, antibody appearance, and symptoms can be described based on observational studies in humans and experimental infection of chimpanzees. Typically in chimpanzees, HCV RNA is usually detectable in the blood within 2 weeks after HCV inoculation (168). The viral level then quickly rises until onset of hepatitis and activation of host immune response, when viral levels may decrease, fluctuate, or become intermittently positive until either complete viral clearance is achieved or the animal progresses to chronic infection. Once chronicity is established, the viral levels remain relatively stable. Similar patterns are seen in human HCV infection, although viral levels may have much wider fluctuations. Following HCV exposure, patients may have intermittently low-level viremia for a period that may last as long as 2 months but is usually approximately 2 weeks (169). This is followed by a ramp phase lasting 8 to 10 days in which viral levels increase rapidly in an exponential manner with a doubling time of 11 hours. Viral levels then reach a high-titer plateau for about 40 to 60 days. Serum ALT levels, marking liver injury, increase during the plateau phase, typically reaching a peak 7 to 8 weeks after infection. Symptoms and jaundice may appear at that time, although commonly these elevations are asymptomatic and thus often missed. Antibodies against HCV appear shortly after ALT starts to increase and their appearance coincides with a decrease in viremia and either resolution or development of chronicity.

Patterns of Virus Replication

The selective infection of human hepatocytes by HCV is probably attributable to viral interaction with cell-surface molecules specifically expressed on liver cells. Viral replication in extra-hepatic sites has, on the other hand, been inconsistently demonstrated (170). In peripheral blood mononuclear cells (PBMC), negative strand RNA has been detected (albeit in a minority of cells) in dendritic cells and B-lymphocytes. It has also been suggested that HCV is able to replicate in the central nervous system, possibly in microglia (171).

Quasispecies distribution, and perhaps subgenotypic distribution (172), appears to differ between the hepatic, serum, and the extra-hepatic compartments. PBMC-specific mutations and amino-acid changes occur in the E1-HVR1 region (170) and the IRES region in the 5′ UTR (172). Similar findings were reported from clones detected in the central nervous system (171). This may reflect strains with tropism for different organs or emergence of tissue-specific adaptive mutations. The clinical significance of these observations is not clear. The detection of HCV RNA in PBMCs of patients who were treated successfully and are free of viremia and liver disease for a long period, as well as the absence of PBMC-specific strains in the serum of the same patients, all suggest that even if extrahepatic replication occurs in PBMCs, it is associated with low viral replication and perhaps failure to mature or export the virus.

Factors in Disease Production

The basic histopathologic findings in chronic HCV infection are similar to those of other chronic viral hepatitides (detailed in chapter 5) and can be separated into inflammatory and fibrotic components, thought to reflect the disease activity (“grade”) and cumulative damage (“stage”), respectively (173). The characteristic inflammatory component consists of a chronic, predominantly monocytic, cellular infiltration of the portal tracts, which can extend into the limiting plate of hepatocytes bordering the tract (“piecemeal necrosis”) and can be accompanied by varying degrees of

necroinflammatory changes in the lobules. Not all components are seen in every biopsy, and these findings probably represent varying degrees of severity. Fibrosis begins by accumulation of extracellular matrix and expansion of the portal tracts. This is followed by formation of septae, which are fibrotic bridges connecting vascular tracts and are mostly porto-portal but also porto-central. Progressive accumulation of these fibrotic bands distorts the liver architecture, and when coupled with regeneration and formation of nodules, is defined as cirrhosis, the end-point of most chronic liver diseases. Various scales have been developed to semi-quantitatively describe those changes. Some histopathologic characteristics, more common in chronic hepatitis C than other chronic viral hepatitides, include the presence of lymphoid aggregates or follicles in the portal tracts (Figure 8a), inflammatory damage to small bile ducts with reactive changes, mild iron overload, and, occasionally, histological findings similar to autoimmune hepatitis with a predominantly plasma-cell infiltrate.

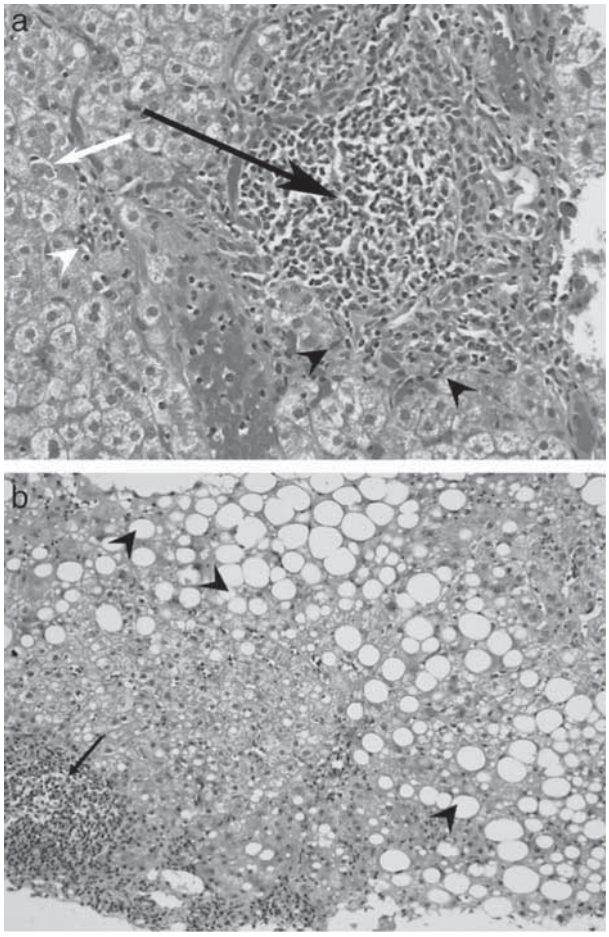

FIGURE 8 Histopathologic findings in chronic hepatitis C. (a) Moderate inflammatory activity. The portal area is expanded by an inflammatory infiltrate and a lymphoid follicle (arrow). The infiltrate disrupts the limiting plate between portal area and hepatic parenchyma ("interface hepatitis", arrowheads). Foci of lobular inflammation can also be seen (white arrowhead) as well as an acidophil body (white arrow). H&E stain, 400× magnification. (b) HCV-associated steatosis. The inflammatory infiltrate (arrow) is accompanied by fat droplets in hepatocytes (arrowheads). H&E stain, 200× magnification. Images provided by David Kleiner, MD PhD, National Cancer Institute, Bethesda, MD.

Steatosis, the accumulation of fat in the hepatocytes, is commonly observed in chronic hepatitis C (Figure 8b), particularly in cases of infection with genotype 3. In non-genotype 3 cases, steatosis mostly reflects the presence of the metabolic syndrome or its components ("metabolic" steatosis), while for genotype 3, steatosis is probably a direct consequence of the viral infection ("viral" steatosis) (174) and appropriately disappears following successful viral eradication (175). The presence of steatosis is associated with accelerated fibrosis and possibly with reduced responsiveness to antiviral therapy.

Immune Responses

HCV, capable of causing chronic infection in most infected individuals, has multiple mechanisms of evading the immune system. Thus, the discussion of the host's immune response is coupled here to discussion of viral evasion mechanisms (Figure 9).

Nonspecific Immune Responses

The single-strand RNA genome of hepatitis C contains numerous secondary-structure components. Upon infection of hepatocytes, these secondary structures are detected by toll-like receptor 3 (TLR3) and retinoic-acid inducible gene I (RIG-I), the intracellular detectors of double-stranded RNA (dsRNA). This initiates a cascade of kinase activation that results in induction of interferon-β expression and its secretion to the surrounding milieu. Interferon-β then acts in an autocrine and paracrine manner, and through dimerization of the type I interferon receptor, activation of Jak1/Tyk2 and of the STAT system induces the transcription of multiple interferon-stimulated genes (ISGs) that confer an antiviral state. HCV has developed several strategies of evading this pathway at multiple levels (176). The afferent arm of the host response, upstream of interferon induction, is effectively blocked by the NS3/4A serine protease, which cleaves and inactivates CARDIF (53) and TRIF (54), the downstream mediators of RIG-I and TLR3, respectively. The interferon-signaling pathway is inhibited by induction of the inhibitory proteins SOCS1 and SOCS3, and by hypomethylation of STAT1, which attenuates its function. Finally, the NS5A and E2 viral proteins inhibit PKR, an interferon-stimulated protein that promotes an antiviral state.

An increased pre-treatment hepatic expression of several ISGs was found in patients who subsequently failed interferon-based treatment (177). Similarly, plasma levels of IP-10 (CXCL-10), another ISG, were higher in patients with a slow response to interferon treatment (178). This observation suggests a maximal, but ineffective, activation of the type 1 interferon pathway in response to HCV infection in some patients. This phenotypic observation has been linked to several single nucleotide polymorphisms in the locus of IFN-lambda genes and point to the importance of this class of interferons in host response to and pathogenesis of HCV infection (179, 180).

Natural killer (NK) cells, the cellular effector arm of the innate immune system, are also important in HCV clearance, chronicity, and pathogenesis of liver damage. NK cells are held under a constant inhibitory influence by interaction of self MHC class I ligands with killer cell immunoglobulin-like receptors (KIRs) on their surface. A specific HLA-C/KIR genotype combination, which has a weaker inhibitory effect on NK cells, increases the rates of clearance of acute HCV infection in patients infected with a small inoculum

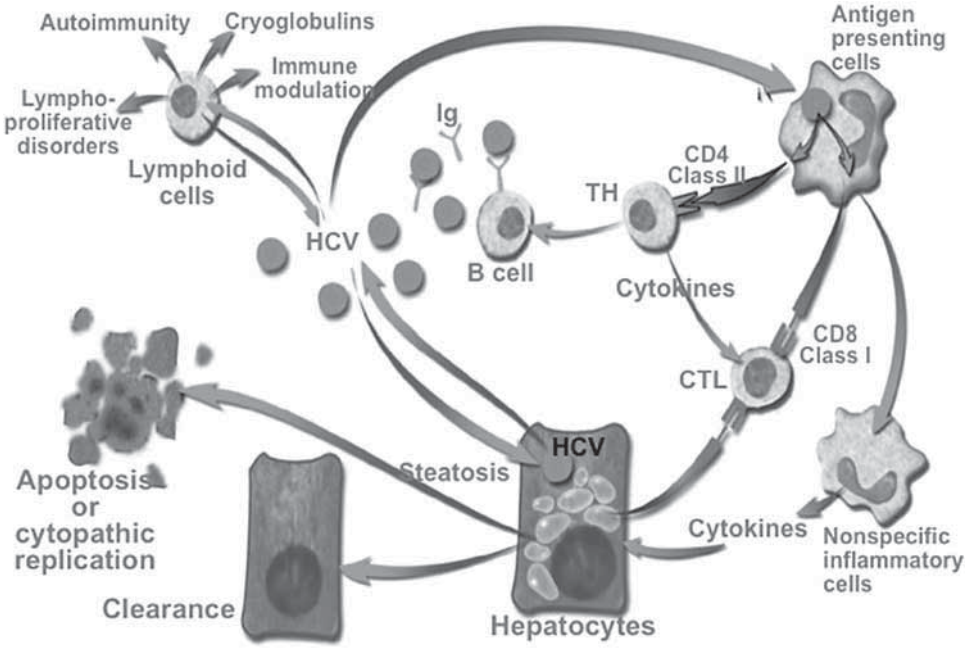

FIGURE 9 The immune response to HCV infection. Hepatitis C infection induces innate and adaptive immune responses. Induction of HCV-specific cytotoxic T-cells by antigen presenting cells is crucial in viral clearance and prevention of chronic infection, through both cytopathic and noncytopathic mechanisms. Antibodies against HCV, secreted by B-cells, appear late and do not seem to have an important role in viral control. Activation of nonspecific inflammatory cells causes liver injury. Chronic stimulation of lymphoid cells can induce autoimmunity and lymphoproliferative disorders including cryoglobulinemia and lymphoma.

(181). This suggests an important role for NK cells in prevention of chronicity. The interaction of NK and dendritic cells is important for dendritic cell (DC) maturation and efficient antigen presentation, which is crucial for recruitment of the adaptive cellular response. The E2 protein of HCV seems to be able to inhibit NK cell activity directly by cross-linking of CD81 receptors on the NK cell surface, and this may be a crucial link in the development of the defective adaptive response discussed below (182).

HCV-Specific Humoral and Cellular Immune Responses

The adaptive immune response against HCV tends to be weak and slow in onset as compared to other viral infections (183). Upon acute infection, a specific cellular response appears after 4 to 12 weeks and is correlated with an increase in liver enzymes, marking the cellular immune response as the likely cause of hepatocyte injury, as opposed to viral cytopathicity *per se*. In general, patients who are able to mount a broad and vigorous $CD4^+/CD8^+$ response tend to recover spontaneously, while patients who become chronically infected have a late, transient, or narrowly focused and weak response. This defect in cellular immunity is HCV-specific and is not associated with a wider immune deficiency. Moreover, the virus-specific T-cells seem to lose their ability to proliferate and produce cytokines over time during chronic infection (184).

Several mechanisms have been proposed to explain the attenuated cellular response. First, inhibition of NK cells and impaired DC maturation can lead to ineffective antigen presentation to the T-cells. Second, continuous exposure to the persistent viral antigenemia may be the cause of the observed $CD8^+$ cell functional exhaustion. Third, direct viral inhibition of T-cells may occur; the viral core protein binds to C1qR on these cells, thereby reducing their function and cytokine secretion. Fourth, escape mutations can develop as a result of the $CD8^+$ T-cell selective pressure (185). And finally, increased HCV-specific regulatory T-cells in the livers of patients with chronic hepatitis C may be responsible for the down-regulation of T-cell response. Irrespective of the mechanism, it seems that the inability to mount and sustain an efficient $CD4^+$ response is the hallmark of progression to chronic infection.

HCV-specific antibodies also appear late during the course of acute infection, typically more than 4 weeks after initial infection. Although antibodies against epitopes in the envelope proteins have been demonstrated to protect from infection in a chimpanzee model, these "neutralizing" antibodies are not protective in humans (183). In fact, the titer of these antibodies is highest in patients with chronic HCV infection, and such antibodies are often absent in patients who resolve infection, probably reflecting the rapid emergence of viral escape mutants during active replication (185). In general, anti-HCV antibodies do not seem to play a major role in the pathogenesis, clearance, and chronicity of hepatitis C.

Correlates of Immune Protection and Disease Resolution

The presence of cellular and humoral immune responses against HCV does not provide full protection from re-infection. Chimpanzees who recovered from HCV infection can be experimentally re-infected, although the recurrent disease seems to be attenuated in severity. This attenuation is dependent on the presence of both memory $CD8^+$ (186) and $CD4^+$ T-cells (187). Re-infection has been reported in human patients in high-risk groups such as active drug users, multi-transfused children with thalassemia and MSM (188,

189). Re-infection is possible after spontaneous recovery, after successful antiviral treatment, and even during treatment. IV drug users who spontaneously cleared viremia are less likely to be infected again upon re-exposure and have lower rates of persistence to chronicity when compared to previously non-exposed controls, as long as they are HIV negative, again suggesting some degree of protection from acquired cellular immunity (190).

Patients who recover from hepatitis C infection have persistent serum antibodies against HCV for a long duration, possibly for life, although a waning titer and sometimes disappearance have been documented after many years. Specific T-cell responses against HCV epitopes also seem to persist for life after spontaneous recovery from acute HCV infection.

CLINICAL MANIFESTATIONS

Major Clinical Syndromes: Acute Hepatitis C

Approximately 3 to 4 million new HCV infections occur worldwide annually (191). Following infection with HCV, most patients develop intermittent viremia and elevated aminotransferases. Symptoms, however, are usually absent or very mild, and thus most cases do not come to medical attention (192). Some patients develop symptoms of hepatitis, which may include nausea, loss of appetite, or jaundice. Only 20 to 50% of patients with acute hepatitis C will resolve, usually within 6 months (median of 16.5 weeks) (Figure 10) (193, 194). In those that do not resolve after 6 months, the chance of spontaneous clearance is low (194). The percentage of resolving cases is a rough estimate, as many cases of acute asymptomatic disease go unnoticed, and is mostly based on serologic cross-sectional studies (195). Young age, female sex, host genetic polymorphisms (especially the IL28B gene), and presence of symptoms seem to be associated with resolution, and so is the vigor and breadth of the immune response (193, 194, 196). Conversely, immune suppression is associated with higher rates of chronicity.

Chronic Hepatitis C

Approximately 170 million people, or 3% of the world's population, are chronically infected with HCV (197). Chronic hepatitis C (CHC) is usually associated with mild, vague symptoms, if any. The most common symptom reported is fatigue, experienced by 50 to 75% of patients and is associated with age, female sex, and advanced disease (198). A vague, right upper quadrant, pain is sometimes associated with CHC. Other associated symptoms may include arthralgia (23%), paresthesia (17%), myalgia (15%), pruritus, (15%), and sicca syndrome (11%). Some patients present only with symptoms of advanced liver disease, such as jaundice, ascites, or gastrointestinal hemorrhage. Physical findings are usually absent unless cirrhosis is present, in which case, jaundice, splenomegaly, spider angiomata, and other cirrhosis-associated manifestations may be seen.

Mild elevation of aminotransferases is commonly observed, predominantly of alanine aminotransferase (ALT), although up to one-third of asymptomatic patients have persistently normal enzymes (199). Antibodies against HCV, as well as relatively stable levels of serum HCV RNA, are universally present.

As discussed above, liver histology in CHC generally consists of inflammatory infiltrate and some degree of fibrosis, ranging from minimal expansion of portal tracts to cirrhosis. The fibrotic process, driven by liver inflammation, can progress over time, although patients differ greatly in rates of progression. In some patients (non-progressors), no increase in fibrosis is seen over decades of follow-up, while in others, it may progress rapidly to cirrhosis within a few years (200). Since the hepatic complications of CHC are generally limited to cirrhotic patients, defining predictive factors that affect the rate of progression, and thus prognosis, is essential (201). The most important predictors of rapid progression are immune suppression, alcohol consumption, male sex, older age at infection, obesity, and degree of histological activity. Estimates of the percentage of patients who progress to cirrhosis range from 4% to 22% at 20 years of infection, depending on methodology and patient population (202).

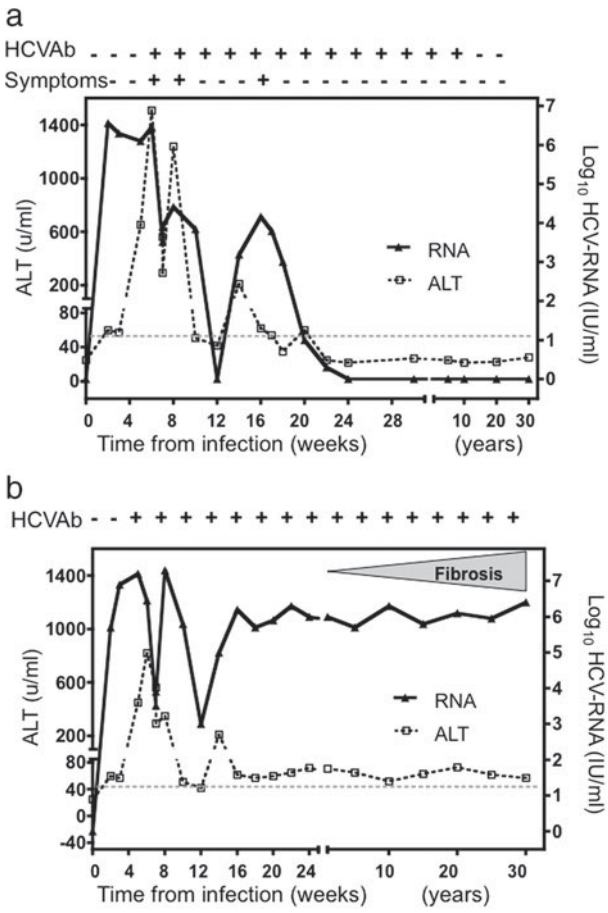

FIGURE 10 The clinical course and natural history of HCV infection. (a) Acute, spontaneously resolving hepatitis C. The liver enzyme elevation, symptoms, and appearance of antibodies usually lag behind viremia. Seropositivity persists for years after recovery but may decline after decades. (b) Acute infection progressing to chronic hepatitis C. Viral levels and enzyme elevations are relatively stable in the chronic phase. Accumulation of fibrosis occurs gradually, over many years. The gray dashed line represents the upper limit of the norm for ALT. HCVAb—anti-HCV antibodies. ALT—alanine aminotransferase.

Acute Liver Failure

Few cases of acute liver failure attributable to HCV have been reported (203). Acute HCV infection causing liver failure appears to be very rare; for example, none of the 308

cases observed by the acute liver failure study group in the United States (204) were caused by HCV. A few cases were reported in patients with CHC, developing fulminant liver failure upon withdrawal of immunosuppressive or chemotherapeutic treatment for other disorders. Fulminant liver failure can also be seen in patients chronically infected with HCV who suffer an acute infection with hepatitis A (205) or B (206), although the association with acute hepatitis A has been debated.

Extrahepatic Manifestations

Although hepatitis C is mostly hepatotrophic, extrahepatic manifestations are not uncommon, presenting clinically or solely as laboratory abnormalities. Some are immunologic phenomena while others may reflect actual viral presence in the affected organ. In a large, cross-sectional study (207), 39% of HCV-infected patients had at least one extrahepatic manifestation, most notably various skin disorders, arthralgias, sicca syndrome, and peripheral neuropathy. Autoantibodies, mostly antinuclear antibodies or rheumatic factor, can be detected in 70% of tested sera. Anti-thyroid antibodies are often detected in HCV patients and can be associated with autoimmune thyroid disease,

A common extrahepatic manifestation is HCV-associated mixed cryoglobulinemia (MC, previously termed essential mixed cryoglobulinemia or type II MC). The hallmark of this immunological disorder is the presence in the serum of a cryoprecipitating monoclonal IgM directed against a polyclonal IgG. High concentrations of HCV can be detected within the cryoprecipitate. Low levels of cryoglobulins can be detected in up to 59% of infected patients (208), mostly without clinical manifestations. However, 10% of patients develop small and medium-sized vessel leukocytoclastic vasculitis manifesting as palpable purpura, arthralgias or arthritis, peripheral neuropathy, glomerulonephritis, and occasionally involvement of other organs. The pathogenesis of HCV-associated MC is assumed to be chronic inflammatory stimulation of B-cells with the subsequent formation of a clonal lymphoproliferative disorder and at times, non-Hodgkin's lymphoma (209). Interferon therapy reduces cryoglobulin levels and decreases symptoms during treatment, which is durable only if sustained virological response is achieved. It is currently unknown if the same effect can be achieved with direct-acting antiviral (DAA) therapy for HCV given the mixed case reports and lack of clinical studies (210, 211).

Apart from MC-related purpura, other skin manifestations of chronic hepatitis are pruritus, porphyria cutanea tarda, and lichen planus (208). Autoimmune thyroid disease is also commonly seen. Membranoproliferative glomerulonephritis, with or without cryoglobulins, is the most common renal manifestation of HCV infection. Sjögren-like lymphocytic sialoadenitis is histologically present in three-quarters of patients with hepatitis C, although only a minority have symptoms of the sicca syndrome. Diabetes mellitus type 2 is more frequent in patients with CHC than with control patients or patients with chronic hepatitis B. This may be related to the impaired glucose tolerance seen with cirrhosis, as well as to insulin resistance promoted directly by HCV.

Disease in Children

Hepatitis C infection in children is usually acquired vertically, and has been reported to occur in 2 to 8% of HCV mono-infected mothers, and perhaps higher in those co-infected with HIV (212, 213). Prior to the era of universal testing of blood products, transfusion-associated disease accounted for many cases of HCV infection, especially in children with inherited coagulation disorders. Today, it is generally accepted that factors associated with vertical transmission include a high viral load, maternal blood exposure from lacerations during vaginal delivery, prolonged rupture of membranes, and HIV co-infection (212). Current evidence does not support the use of cesarean section to reduce the risk of vertical transmission. In those infected with HCV, during the first 2 decades of life, the disease seems to be mild and asymptomatic, although cirrhosis and complications have been reported (214). Spontaneous clearance of infection in vertically infected children can be seen in 20 to 25% of patients and can occur as late as age 3 years, and occasionally even later. Of the remaining 80%, non-invasive studies suggest mild asymptomatic disease in 50% and active disease in 30% (148). However, liver biopsies, although usually demonstrating mild disease, can also demonstrate progression of fibrosis with prolonged disease duration (215). Thus, although symptomatic disease is not commonly seen in children, it is not clear whether this reflects a true difference in disease behavior from the adult population, or is just reflective of the shorter duration of disease.

Disease in Immunocompromised Hosts

Immune suppression has a significant effect on the natural history of HCV infection. Co-infection with HIV is common, and it is estimated that 30% of HIV infected patients in the United States and Europe are co-infected with HCV, with even higher rates in hemophiliacs and IV drug abusers (216). Rates of co-infection in Africa appear to be much lower (217), perhaps because the dominant route of transmission for HIV is sexual in these regions. Since the advent of HAART, with prolonged survival of HIV-infected patients, liver disease has emerged as a leading cause of mortality, ranking third only to AIDS-related death and non-AIDS-defining cancers (218). Co-infection is associated with higher serum HCV RNA levels, worse histopathological findings, and accelerated rates of fibrosis. Cirrhosis, HCC, and death from liver disease tend to appear after a shorter duration of infection in co-infected patients. HAART treatment can slow the progression of liver fibrosis, although the rates of drug-hepatotoxicity are increased in co-infected patients. In the past era of interferon-based therapies, treatment success rates were lower in co-infected patients with treatment results directly related to $CD4^+$ counts (219). However, with direct-acting antiviral (DAA) therapy, co-infected patients now experience similar success rates compared to those who are mono-infected (220).

Organ transplantation, and its associated prolonged immune suppression, can also affect the progression of HCV infection. Patients in need of organ transplantation historically have had higher rates of HCV infection, probably because of the need for blood products and for multiple, invasive, therapeutic, and diagnostic interventions. This is especially true for kidney transplant recipients, for whom hemodialysis is a major risk factor and on whom more data is available. Serum HCV RNA levels increase after kidney transplantation, and this was particularly prominent after a course of anti-lymphocyte antibodies. HCV infection portends worse outcome after kidney transplantation, with decreased graft- and patient-survival, although most deaths are not due to liver disease (221). The limited data on progression of the liver disease in HCV-infected kidney transplant recipients suggest more advanced fibrosis in these patients (222). In hematopoietic stem-cell transplant recipients,

hepatitis C seems to progress more rapidly to cirrhosis compared to non-transplanted patients (223), and pre-existing hepatitis C with elevated enzymes increases the risk for severe veno-occlusive disease, a serious hepatic complication of stem cell transplantation (224). Curiously, cancer chemotherapy and its associated profound immune-suppression may be associated with minor liver enzyme elevations, but rarely with serious flares of hepatitis C.

Liver transplantation for hepatitis C is distinctive, since the infected organ itself is removed and is replaced by an uninfected organ. Re-infection of the graft is universal and is associated with recurrence of hepatitis and cirrhosis in a significant number of patients, resulting in poorer graft and patient survival when compared to patients who had a liver transplantation for indications other than hepatitis C (225). A significant factor affecting survival is the need for immunosuppressive treatment of acute rejection episodes, with bolus steroids, lymphocyte-depleting agents, or anti-IL-2 receptor antibodies. In fact, maintaining a stable immunosuppressive dose and avoiding rapid changes were shown to improve outcome significantly (226).

Complications

Decompensation and complications may complicate the cirrhosis of CHC. These complications are not unique to infection with HCV and can be seen with other etiologies of cirrhosis. The complications include hypersplenism, bleeding from esophageal or gastric varices, portal hypertensive gastropathy, hepatic encephalopathy, ascites, spontaneous bacterial peritonitis, renal failure, and death. Not all cirrhotic patients are symptomatic or present with complications. Decompensation occurs at an estimated rate of about 4% per year (227, 228).

Hepatocellular carcinoma (HCC) can develop as a complication of cirrhosis, irrespective of the etiology, although some etiologies are associated with a much higher likelihood of HCC. The yearly incidence of HCC in patients with cirrhosis due to HCV infection is estimated at 2 to 8% (229). The risk for non-cirrhotic patients appears to be much lower, although underestimation of the severity of the underlying liver disease may lead to false assurance in these patients. As discussed below, successful eradication of hepatitis C with antiviral treatment significantly reduces, but does not eliminate completely, the risk of HCC.

Differential Diagnosis

Diagnosis of chronic hepatitis C is straightforward when the appropriate laboratory tests are available. The differential diagnosis of elevated liver enzymes is vast; other causes of infectious hepatitis are discussed in chapter 5. However, a positive serum HCV antibody is almost always a marker of exposure to HCV and a positive serum HCV RNA test is diagnostic for the disease. These tests should be performed on all patients with elevated aminotransferases, and can be used to screen patients with high-risk behaviors (injection drug use or intranasal illicit drug use), high-risk exposures (long-term hemodialysis, healthcare exposures, incarceration, and individuals with HIV), and blood or organ donors. In addition to these patients thought to be at high risk for HCV infection, the Centers for Disease Control and Prevention (CDC) and the United States Preventive Services Task Force recently recommended expansion of risk-based HCV screening guidelines to include a one-time HCV screening in all persons born during 1945–1965, which is a birth cohort that has been demonstrated to have a higher risk of having HCV infection (230, 231).

LABORATORY DIAGNOSIS

Virus Detection

Nucleic acid testing is the gold standard for detecting active HCV replication. Detection of viral genome sequence in the plasma, quantitatively or qualitatively, is typically performed using automated assays with sensitivity and specificity nearing 100% (232). Qualitative assays to detect the presence of hepatitis C most commonly utilize reverse transcriptase-polymerase chain reaction (RT-PCR) assays and have a lower detection limit of 50 international units (IU)/ml. Assays based on the transcription-mediated amplification (TMA) method are even more sensitive, with a lower threshold of 5 IU/ml. In most clinical scenarios, a qualitative result is sufficient for the diagnosis and monitoring of patients with chronic and acute hepatitis C. Quantitative measurement of HCV viral load is required before and during treatment of patients to assess their response and can be performed using quantitative real-time RT-PCR. The 2nd quantitative real-time RT-PCR has a high dynamic range and a lower limit of detection of 15 IU/mL. To facilitate standardization, the WHO developed a panel of standard calibration samples and promoted the use of the arbitrary international units. The conversion from copies/ml to IU/ml differs based on the commercial assay used, and ranges from 0.9 to 5.2 copies/ml to 1 IU/ml equivalent.

An assay for detection of an HCV core protein antigen (233) incorporates an immune complex dissociation step (to diminish interference from naturally occurring anti-core antibodies) followed by an enzyme immunoassay (EIA) step. The assay has a detection threshold of 20,000 IU/mL with specificity of more than 99%. Despite the excellent performance of the assay, it is still inferior to nucleic acid detection assays, which becomes positive earlier during the window period.

Serologic Assays

Serologic assays for detecting HCV infection were rapidly developed and improved following the initial discovery of the virus because of the urgent need to screen blood donors and prevent transmission. The first EIA test contained a single recombinant antigen derived from the NS4 protein and was limited in both sensitivity and specificity. A second generation test, with additional antigens from the core and NS3 proteins, and a third generation test, with reconfigured core- and NS3-based antigen as well as an antigen from NS5, markedly improved test performance and shortened the window period between infection and seroconversion. The currently available third generation assays, especially those utilized in the western world, provide sensitivity of 100% and specificity of nearly 100% when tested against standardized panels. As opposed to their use in diagnosing chronic hepatitis C in a clinically suspected case, the positive predictive value of the EIA tests is lower when they are used for screening of a low-prevalence population, such as blood donors, and often requires a confirmatory test. In the past, a recombinant immunoblot assay (RIBA) could be used to confirm a positive EIA in this setting, although the nucleic acid tests discussed above are superior and RIBA is no longer available. With the current sensitivity and specificity of the EIA assays, a confirmatory test may no longer be necessary in the context of screening a low-risk population (234).

In 2010, the U.S. Food and Drug Administration (FDA) approved the use of a rapid diagnostic test that utilizes immunochromatography to identify HCV antibodies in oral fluid and blood (serum, plasma, or whole blood). This has

facilitated the possibility of point-of-care (POC) testing to improve turnaround time and the making of rapid clinical decisions. While many POC tests are under clinical evaluation, there is currently only one rapid HCV test approved for clinical use by the FDA, which has a >90% sensitivity and >99% specificity for detecting antibodies to HCV. Since these rapid tests are used to identify patients with HCV antibody, they would have limited utility in diagnosis of acute hepatitis C given that HCV antibody can only be detected in 80% of patients after 15 weeks of exposure.

PREVENTION

General

Persons infected with HCV should be advised to avoid sharing toothbrushes, razors, nail clippers, and other personal care articles that may become contaminated with blood (235). Patients in a long-term heterosexual monogamous relationship need not alter their sexual practices, although testing the partner for exposure and counseling on the potential risk is advised. Couples may consider the use of barrier protection to reduce the already low risk, or during higher risk sexual practices such as anal sex or intercourse during menses. Persons involved in high-risk sexual behavior (multiple partners, violent sex, sex workers, etc.) should be advised to use barrier protection to prevent HCV transmission and infection with other sexually transmitted diseases (152). Drug users should be counseled to avoid sharing of needles, syringes, and other injection and snorting paraphernalia. Needle-exchange programs have been shown to reduce HCV seroprevalence.

Since alcohol consumption is associated with acceleration of liver injury and fibrosis, minimizing alcohol intake should be recommended for all patients. Routine vaccination against hepatitis A and B viruses should be done to reduce the risk of superinfection and potential liver failure.

Passive Immunoprophylaxis

Passive immunoprophylaxis against HCV reinfection has been attempted in the context of liver transplantation, in light of the success in the use of hepatitis B immunoglobulin to prevent HBV reinfection of the graft. Anti-HCV-enriched human immunoglobulin preparation failed to prevent re-infection in HCV patients after liver transplantation (236). The use of a monoclonal anti-E2 antibody in this setting in a phase 2, randomized, controlled trial had only a transient effect on viral levels (237). This same antibody demonstrated short-term (less than 48 hours) reduction of viral levels when administered to non-transplanted patients with chronic hepatitis C (238). With further development of broadly neutralizing antibodies, it is possible that passive protection may still be achievable (239–241).

Active Immunization

Despite remarkable recent advances in the understanding, testing, and treatment of HCV, the field of prophylactic vaccine development for HCV has lagged significantly behind. Although therapies for HCV are rapidly evolving, the development of a vaccine remains paramount given the current global public health burden of HCV, current and projected future cost of care, and lack of accessibility of care in less-industrialized countries. Over the past few decades, many attempts have been made to develop an HCV vaccine; however, all have failed at various stages of clinical development (242). The difficulty in designing an effective HCV vaccine is multifaceted, including: (i) an incomplete understanding of the mechanisms of protective immunity in HCV infection (242); (ii) design of a vaccine that can encompass the HCVs worldwide genetic diversity(243); (iii) HCVs ability to hide from the antibody response(244); (iv) HCV's ability to alter the activation of T-cells with resultant ineffective cell-mediated immunity (184, 245); and (v) the lack of convenient and authentic experimental animal model systems for vaccine development (246).

Currently, various candidate vaccines are in different stages of clinical development. While many candidate vaccines have utilized various viral vectors including adenovirus, vaccinia virus, modified vaccinia Ankara (MVA), fowl pox viruses, and other viruses with the aim of delivering various HCV structural and nonstructural antigens for induction of T-cell-mediated immunity, the adenoviral vector seems to be the most promising due to their ability to induce robust $CD4^+$ and $CD8^+$ T-cell responses with a predominant T_H1 phenotype (247–250). Other vaccine vehicles include HCV viral proteins, DNA immunization, and virus-like particles (VLPs) that induce both humoral and cellular arms of the immune response and/or peptides that target specific T-cell epitopes and biological adjuvants that hope to have efficacy in humans. With continued efforts in the field of HCV vaccination, the hope is that this field will catch up to the treatment field to provide a two-pronged attack for global eradication of HCV infection in humans.

Antiviral Chemoprophylaxis

As discussed below, in documented cases of acute HCV infection, antiviral treatment is withheld for 8 to 16 weeks to allow the patient a chance to clear the virus on their own. Because interferon and ribavirin are expensive and fraught with side effects, chemoprophylaxis after exposure or in high-risk populations is not advised. However, with the approval of highly effective oral antivirals against HCV, post-exposure prophylaxis with DAAs has yet to be explored and will be an interesting topic to revisit in the future. Additionally, the use of DAAs as prophylaxis during the anhepatic phase of liver transplantation has only been described in a case report and formal investigation is still lacking (251).

Management of Outbreaks

Outbreaks of hepatitis C associated with a single source of transmission are often detected late as most cases of acute infection are asymptomatic and go unnoticed. Iatrogenic outbreaks as a result of contaminated medical or dental instruments or contaminated injections/infusions have been reported (137, 139, 252, 253). Recently, outbreaks among communities of MSM and persons who inject drugs (PWID) have been reported to be on the rise and appear to be the major route of HCV transmission in the developed countries (254). When such an outbreak is detected, screening of all potentially affected patients, reviewing of potential breaches of standard care, and offering of treatment to infected individuals should be undertaken.

Prevention of Perinatal and Congenital Infection

Although HCV can be transmitted vertically, there is no effective method to prevent transmission. Caesarean section was not proven to prevent infection of the newborn and should not be recommended routinely for HIV-negative mothers (150). Invasive fetal monitoring during pregnancy appears to be associated with increased risk of transmission (147). Thus, it is prudent to avoid invasive monitoring if possible without compromising maternal or fetal safety.

TREATMENT

Indications for Therapy and Monitoring of Response

In general, antiviral treatment is indicated for all patients with chronic hepatitis C who are viremic, except those with significant co-morbidity due to non-liver related conditions (255). Normal aminotransferase levels should not exclude patients from therapy, as up to one-third of patients may have advanced disease on histopathology. The goal of therapy in those infected with HCV is a reduction in all-cause mortality and liver-related adverse health consequences. A liver biopsy, while invasive, is the diagnostic gold standard and is helpful in determining the degree of disease severity to provide prognostic information. More recently, the use of noninvasive fibrosis biomarkers utilizing indirect (routine tests) or direct (components of extracellular matrix produced by activated hepatic stellate cells) serum biomarkers and vibration-controlled transient elastography has gained popularity and is an acceptable substitute in assessing fibrosis in chronic HCV infection (255, 256). Based on available resources, it is currently recommended that patients with advanced fibrosis, compensated cirrhosis, liver transplant recipients, and those with severe extrahepatic HCV manifestations should be considered as high priority for therapy (255). However, there is emerging evidence that demonstrates a greater benefit in patients who are treated at an earlier stage of hepatic fibrosis (257–259).

Response to therapy, assessed by quantitative RT-PCR, is the standard-of-care during and after therapy. A sustained virological response (SVR) is defined as a negative HCV RNA test after a defined time period after completion of therapy. Achieving an SVR, inherently a virological outcome, is an accepted surrogate marker for beneficial clinical outcomes, and has been equated with "being cured of HCV" in the public domain. Those that are persistently HCV RNA negative post-therapy are less likely to progress to decompensated liver disease, to die from hepatic causes, or to develop hepatocellular carcinoma, even if they already have established cirrhosis (260, 261). In general, the likelihood of relapse later than 6 months after treatment (the currently accepted time point for SVR determination) is very low and re-appearance of HCV viremia more frequently indicated re-infection rather than relapse. Although HCV RNA sequences can occasionally be detected by PCR or TMA in peripheral blood mononuclear cells (PBMCs) or liver tissue (see above), this has not been shown to be associated with enzyme elevation or histological worsening of liver disease and the significance of these findings remains unknown. Historically, a SVR was defined as 24 weeks after the end of treatment; however, a large retrospective analysis from five phase III studies, performed in 2015, described a high rate of concordance between 12 and 24 weeks post-therapy (262). An undetectable HCV RNA 12 weeks after therapy is currently accepted by clinicians and regulatory authorities as a primary endpoint of therapy and an SVR.

The Historical Era of Interferon-Based Therapy

The first use of interferon for chronic non-A non-B hepatitis was evaluated at the National Institutes of Health Clinical Center in 1984 and the first patient was cured of HCV with interferon therapy shortly thereafter (263, 264). Interferon induces a multitude of host genes that promote an antiviral state. These seminal pilot studies evaluating interferon for HCV led to the initiation of two randomized controlled trials which subsequently led to FDA approval the use of interferon α-2b for chronic hepatitis C in 1991 (265, 266).

Despite the initial enthusiasm of the use of interferon for chronic HCV infection, this therapeutic modality had an SVR rate of only 6% after 24 weeks of therapy and 13 to 19% with 48 weeks of therapy (267, 268). These SVR rates were even worse when evaluated according to HCV genotype, whereby genotype 1 infection resulted in less than a 2% SVR with 24 weeks of therapy and 7 to 11% SVR rate with 48 weeks of therapy.

The next major improvement with interferon-based therapies occurred in the early 1990s, after ribavirin was noted to have anti-HCV activity (269–272). Subsequent large-scale studies evaluating the combination of interferon-α and ribavirin revealed increased SVR rates to 16% with 24 weeks of therapy and 42% with 48 weeks of therapy (271, 273, 274). Within the next decade, SVRs became associated with favorable long-term clinical outcomes, and the combination of interferon and ribavirin was approved as the standard-of-care for the treatment of chronic HCV.

Thereafter, subsequent improvements to the delivery of interferon were achieved by attaching a polyethylene glycol (PEG) molecule to interferon, thereby prolonging the half-life of interferon and further improving response rates to interferon-based therapies (275, 276). When used in combination with ribavirin, pegylated interferon (peginterferon) induced an SVR in up to 46% of patients with HCV genotype 1 infection and up to 82% in patients infected with genotype 2 and 3 infection (Figure 11) (277, 278). Therefore, peginterferon-α with ribavirin became the standard-of-care for all genotypes of chronic HCV infection only differing by duration of therapy (48 weeks for genotypes 1 and 4 and 24 weeks for genotypes 2 and 3).

Dual therapy with peginterferon and ribavirin (7) demonstrated a biphasic response, with a rapid decline over the first 2 days and a slower, second-phase decline lasting at least up to day 14 of treatment. Mathematical modeling applied to these data suggested that the first phase represented rapid clearance of the virus from serum on the basis of inhibition of replication and of new virion production by interferon. The second phase corresponded to the death and clearance of infected cells in addition to inhibition of viral replication. With this early therapeutic modality, failure to achieve an early virological response (EVR) (defined as negativity or

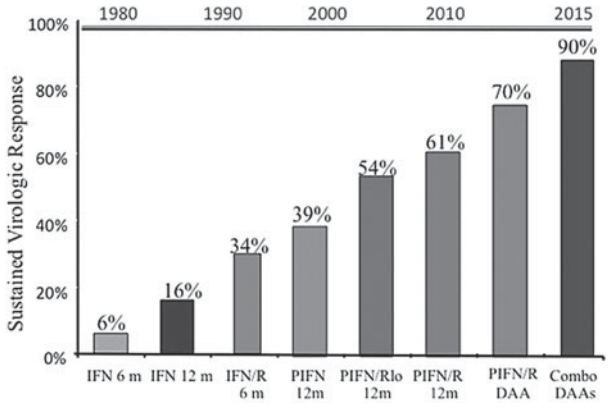

FIGURE 11 The evolution of treatment regimens for chronic hepatitis C and the corresponding rates of sustained virological response. IFN—interferon alfa. PIFN—pegylated interferon alfa. R—ribavirin. DAA—direct-acting antiviral therapy.

more than a 2 log decrease in viral load by week 12) or to become HCV-RNA by week 24 of treatment (279), had a very high (>95%) negative predictive value for response and allowed clinicians to terminate the treatment course early for some patients. Aside from the assessment of on-therapy virological response and infecting genotype as predictive factors of response, a high pre-treatment viral load, African-American race, obesity, advanced fibrosis, and a host IL28B TT genotype were all unfavorable factors for interferon-based therapy.

Along with response rates <50% with interferon-based dual therapy for CHC, the side effects of interferon made this therapeutic modality very difficult for patients to tolerate for 24 to 48 weeks (277, 278). Among the various notable side effects of interferon, hematologic toxicity (especially neutropenia (280) and thrombocytopenia) was the most common reason for dose reduction or interruption and occurred in up to 20% of patients. Interestingly, psychiatric side effects of interferon, especially depression, were more common in HCV patients than in patients with chronic hepatitis B treated with the same doses.

In acute HCV infection, treatment indications and regimens are less standardized. Much of this is due to the lack of large-scale studies for acute HCV infection. Currently, treatment should be deferred for 12 to 16 weeks from the time of infection, to allow for possible spontaneous clearance to occur, especially in symptomatic patients. One randomized phase III study with interferon-based therapy demonstrated non-inferiority between starting therapy at the time of diagnosis versus delaying therapy for 12 weeks after identification of acute infection (281). Treatment can be offered using standard or peginterferon monotherapy. Treatment for genotype 1 infection should probably last 24 weeks while for genotypes 2, 3, or 4, a course of 8 to 12 weeks of treatment seems to be sufficient (282). A high likelihood of treatment success, with SVR rates above 90%, was reported in several series, suggesting that, at least for genotype 1 infection, treatment before chronicity develops should be attempted whenever possible (283).

Ribavirin

Ribavirin (RBV) is a synthetic oral guanosine nucleoside analogue that was initially identified to have broad-spectrum antiviral activity against both DNA and RNA viruses in *in vitro* and *in vivo* models (284). In HCV as monotherapy, it demonstrated improvement in aminotransferase levels but not in decreasing viral load *in vivo* (269, 270,285–288). However, as discussed in the previous section, when utilized in combination with interferon or pegylated interferon, ribavirin significantly improved SVR rates and became the standard-of-care for over 10 years.

Despite the utility of RBV for HCV, the mechanism of action of ribavirin in HCV has never been fully elucidated. Various mechanistic hypothesis have been proposed, including: (i) RNA viral mutagenesis through incorporation of ribavirin triphosphate into the HCV viral genome resulting in nucleotide transitions; (ii) direct inhibition against HCV RNA dependent RNA polymerases leading to inhibition of genome replication; (iii) alteration of the host adaptive immune response through Th2 response suppression and Th1 response induction leading to increased clearance of infected cells; (iv) inhibition of host inosine monophosphate dehydrogenase resulting in decreased synthesis and lower GTP levels and subsequent inhibition of genome replication; and (v) potentiation of interferon activity through modulation of genes involved in interferon signaling and/or an indirect mechanism that may act to reset interferon-responsiveness in an HCV-infected liver (289–295).

Before the current era of direct-acting antiviral (DAA) therapy, ribavirin was administered orally twice daily, dosed according to weight and genotype. Patients with genotypes 1 or 4 infection were treated with peginterferon and weight-based ribavirin (1,000 mg in those <75kg and 1,200 mg in those ≥75 kg) for 48 weeks, while for patients with genotypes 2 or 3, a 24-week course of peginterferon with 800 mg of ribavirin was sufficient (296). In the current era of DAA therapy, weight-based ribavirin therapy still plays a role in certain therapeutic regimens with an improvement in SVR rates (297).

During the era of dual therapy with peginterferon and ribavirin, ribavirin-induced hemolysis played a large role in dosing limitations. The side effects of ribavirin were managed mainly by dose reduction, although this approach was undesirable as significant dose-reduction (>20%) was associated with decreased response rates to treatment (298), especially if dose adjustments were made early in the treatment course.

The Era of Direct-Acting Antiviral Therapy for HCV

With the less-than-optimal response rates to interferon-based therapies, especially for genotype 1 patients, along with the side effects, different therapeutic strategies were sought. The development of new therapeutic targets was promoted by the development of a genotype 1 subgenomic and genomic replicon system and the identification of a genotype 2a JFH1 clone (299). These new model systems allowed for the identification of the steps of the HCV life cycle, further allowing for the development of new therapeutic targets (Figure 12). As a result, in 2011, the first DAA therapies, boceprevir and telaprevir, were approved by the FDA for use as "triple therapy" in combination with peginterferon and ribavirin in patients with chronic HCV genotype 1 infection (300–304). These first-generation DAAs inhibited the HCV NS3/NS4A protease by binding to the active site, thereby inhibiting HCV replication, and significantly increasing SVR rates in HCV genotype 1 infection to 69 to 75% when used as "triple therapy" with peginterferon and ribavirin (300, 301).These DAA regimens quickly became the standard-of-care for chronic HCV genotype 1 infection in 2011 (305). However, these first generation DAAs have a low barrier to resistance, leading to the rapid selection of resistance-associated variants when used either alone or in combination with peginterferon and ribavirin, and were poorly tolerated, especially when used as triple therapy (with serious adverse events and even death), and had low response rates in prior null responders to peginterferon and ribavirin (306, 307).

The development of HCV DAAs has progressed rapidly, and in 2012, several phase II studies demonstrated that it was possible to cure chronic HCV infection without the use of peginterferon-α (308–310). Subsequent phase III studies have confirmed this finding, especially with improved understanding that therapeutic targets can be directed at blocking different steps in the HCV replicative cycle (311, 312). Currently, the therapeutics under drug development include NS3-4A protease inhibitors, nucleotide analogue and non-nucleoside inhibitors of the HCV RNA-dependent RNA polymerase (RdRp), and inhibitors of the non-structural 5A (NS5A) protein (Table 3).

Simeprevir (a NS3/4A protease inhibitor), which was the third DAA to receive FDA approval in 2013 for use in combination with peginterferon and ribavirin for genotype 1

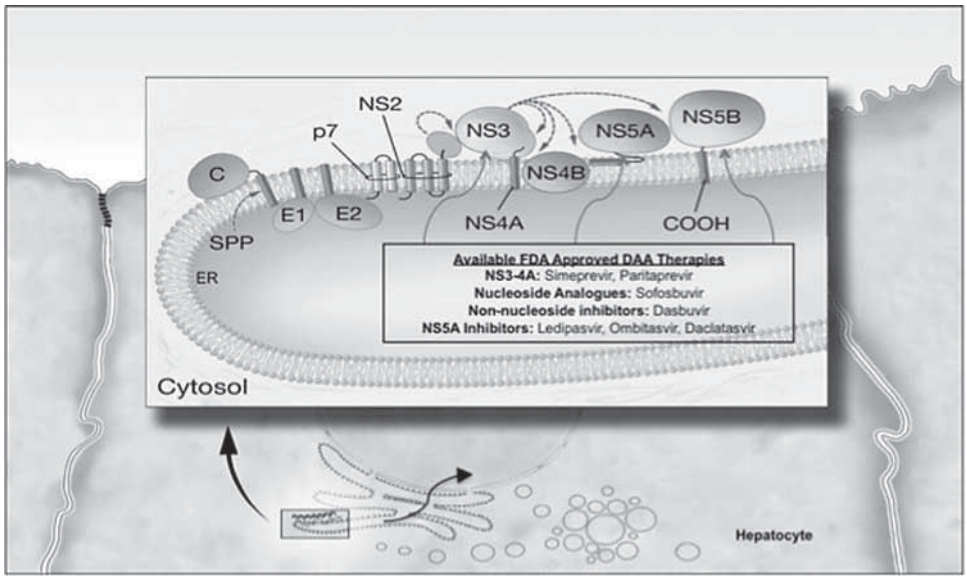

FIGURE 12 Currently FDA approved direct-acting antiviral therapy for hepatitis C includes NS3-4A protease inhibitors, nucleotide analogue, and non-nucleoside inhibitors of the HCV RNA-dependent RNA polymerase (RdRp) and inhibitors of the non-structural 5A (NS5A) protein.

infection. This regimen achieved SVR in 80% of treatment naive patients and in 79% of treatment-experienced relapsers. Two weeks after simeprevir's approval, the first multigenotypic DAA sofosbuvir, received FDA approval for use in genotypes 1, 2, 3, and 4 infection. This drug was the first of the nucleotide analogues to be approved for use in CHC. In genotypes 1 and 4, sofosbuvir was approved for use with peginterferon and weight-based ribavirin for 12 weeks. In genotype 2, sofosbuvir was approved for use with weight-based ribavirin without interferon for 12 weeks and in genotype 3 for 24 weeks. This approval heralded a new era of interferon-free therapies.

HCV Genotype 1 Therapy

In contrast to interferon-based therapies, the paradigm has shifted and genotype 1 is no longer difficult to treat in the current era of DAAs.

Simeprevir and Sofosbuvir Combination Therapy

The combination of simeprevir (a NS3-4A protease inhibitor) and sofosbuvir (a nucleoside analogue) was the second interferon-free DAA regimen approved for use in HCV genotype 1 infection. One phase III study that demonstrated a ≥95% SVR rate in those without cirrhosis regardless of viral subtype (1a or 1b) when treated for 12 weeks (313). Another phase III study demonstrated an overall SVR rate of 83% in cirrhotic patients (88% in treatment-naive patients and 79% in prior non-responders to peginterferon and ribavirin dual therapy patients) and an SVR rate of 74% in genotype 1a patients with cirrhosis (314). Based on the results from these large studies, the combination of simeprevir and sofosbuvir can be administered for 12 weeks in patients without cirrhosis and 24 weeks with or without ribavirin in those with cirrhosis.

Sofosbuvir and Ledipasvir Combination Therapy

In late 2014/early 2015, the combination of the sofosbuvir (a nucleotide analogue) and ledipasvir (an NS5A inhibitor) was approved for use in HCV genotype 1 in the United States and genotypes 1, 3, and 4 in Europe. Phase III studies demonstrated a >90% SVR rate when used with or without ribavirin for 12 to 24 weeks in both treatment-naive and treatment-experienced patients (315–317). In patients with cirrhosis (compensated and decompensated) and those in the pre- and post-liver transplant setting, SVR rates ranged from 85 to 95% depending on the severity of disease (318–321). The combination of sofosbuvir and ledipasvir for 12 weeks of therapy in treatment-naive or treatment-experienced patients without cirrhosis, and 24 weeks in treatment-experienced patients with cirrhosis is appropriate.

Ombitasvir, Dasabuvir, and Ritonavir-Boosted Paritaprevir Combination Therapy

Shortly after the approval of sofosbuvir and ledipasvir, the DAA combination of ombitasvir (an NS5A inhibitor), dasabuvir (a nonnucleoside NS5B polymerase inhibitor), and ritonavir boosted paritaprevir (an NS3/4A protease inhibitor) received FDA approval for the therapy of HCV genotype 1 infection. Phase III studies demonstrated SVR rates >95% in genotype 1a patients when used in combination with weight-based ribavirin and approximately a 90% SVR rate when used without ribavirin. In genotype 1b patients, SVR rates were >97% without the use of ribavirin. Thus, this fixed-dose regimen can be used with weight-based ribavirin for 12 weeks in genotype 1a patients without cirrhosis and 24 weeks in those with cirrhosis. For genotype 1b patients, the fixed dose DAA combination is recommended for 12 weeks without ribavirin.

Daclatasvir and Sofosbuvir Combination Therapy

In July 2015, the FDA approved the use of daclatasvir (an NS5A inhibitor) for use with sofosbuvir for genotype 3 HCV infection. Although this combination was not yet approved for use in genotype 1 infection, existing data from several studies suggested efficacy in genotype 1 infection (255). In one phase IIB study and two phase III studies evaluating daclatasvir and sofosbuvir for 12 and 24 weeks in various genotype 1a and 1b populations (with and without cirrhosis, co-infected with HIV), SVR rates >95% were achieved in genotype 1b infection with 12 weeks of therapy (322–324).

TABLE 3 Direct-acting antiviral therapeutic targets, mechanisms of action, and approved and investigational therapies

HCV target	Mechanism of action	Drug same	HCV genotype activity	FDA status	Status
NS3-4A protease inhibitors	Bind to the catalytic site of the NS3-4A protease and block post-translational processing of the viral polyprotein	Telaprevir	1	Approved	No longer available
		Boceprevir	1	Approved	No longer available
		Simeprevir	1,2,4	Approved	In use
		Paritaprevir	1,2,4	Approved	In use
		Asunaprevir	1,2,4	Not approved	Under development
		Sovaprevir	1,2,4	Not approved	Under development
		Vaniprevir	1,2,4	Not approved	Under development
		Vedroprevir	1,2,4	Not approved	Under development
		ACH-2684	All	Not approved	Under development
		ABT-493	All	Not approved	Under development
		Ciluprevir	All	Not approved	Under development
		GS-9857	All	Not approved	Under development
		IDX320	All	Not approved	Under development
		Grazoprevir	All	Not approved	Under development
Nucleoside/ Nucleotide analogues	Function as false substrates for the HCV RdRP and lead to chain termination after incorporation into synthesized viral RNA	Sofosbuvir	All	Approved	In use
		ACH-3422	All	Not approved	Under development
		AL-335	All	Not approved	Under development
		MK-3682	All	Not approved	Under development
Non-nucleoside inhibitors of the HCV RdRp	Bind to one of four allosteric sites of the enzyme and change the conformation of the RdRp which results in blocking of its catalytic function	Dasabuvir	All	Approved	In Use
		Beclabuvir	All	Not approved	In development
		GS-9669	All	Not approved	In development
NS5A inhibitors	Bind to domain 1 of the NS5A protein, thereby blocking regulation of HCV replication. Also inhibit assembly and release of viral particles	Ledipasvir	1,2,4	Approved	In use
		Ombitasvir	1,2,4		
		Daclatasvir	All		
		Elbasvir	All	Not approved	In development
		ACH-3102	All	Not approved	In development
		ABT-530	All	Not approved	In development
		Odalasvir	All	Not approved	In development
		Velpatasvir	All	Not approved	In development
		MK-8408	All	Not approved	In development
		GS-5816	All	Not approved	In development

However, in those with cirrhosis (genotype 1a or 1b) or genotype 1a infection, SVR rates were only reached in 76% with or without ribavirin. Based on this information, this combination can be administered for 12 weeks in treatment naive genotype 1b patients without cirrhosis whereas 24 weeks of therapy with or without ribavirin can be instituted in treatment naïve genotype 1b patients with cirrhosis or all patients with genotype 1a infection.

HCV Genotype 2 Therapy

With interferon-based therapy, genotype 2 infection was considered as an easy-to-treat genotype. Interferon-free DAA regimens also provide very high response rates; however, some available therapies still require the use of ribavirin.

Sofosbuvir with Weight-Based Ribavirin Combination Therapy

One of the earliest approved interferon-free regimens for chronic HCV infection was for the treatment of those with HCV genotype 2 or 3 infection. Phase II and III studies described an SVR rate of approximately 94% in genotype 2 infection (325–328). Thus, the combination of sofosbuvir plus weight-based ribavirin for 12 weeks has been used in patients with genotype 2 infection with extension to 16 weeks in patients with cirrhosis (255).

Daclatasvir and Sofosbuvir Combination Therapy

In 2015, the combination of daclatasvir and sofosbuvir, while not FDA approved for genotype 2 infection, has been utilized as an off-label therapy. Studies have demonstrated SVR rates >92% in treatment naïve patients with both 12 and 24 weeks of therapy (324, 329). Thus, this regimen has been utilized for 12 weeks in treatment naive patients or those who are unable to tolerate ribavirin therapy.

HCV Genotype 3 Therapy

In the era of interferon-free DAA therapy, HCV genotype 3 has become the "difficult-to-treat" population. Many of the

available DAA therapies are significantly less effective in this population and have a high rate of viral resistance. In fact, in 2015, it was the only genotype for which pegylated interferon was still recommended as a first-line therapy to be used in conjunction with a DAA (255).

Peginterferon, Ribavirin, and Sofosbuvir Combination Therapy

In a phase III, triple-arm, clinical trial, patients treated with the combination of peginterferon, ribavirin, and sofosbuvir for 12 weeks achieved 95% SVR rate compared to rates of 77% for 16 weeks and 88% for 24 weeks of therapy without peginterferon (330). Prior to this study, other studies have described an overall SVR rate of 84%, which was higher in treatment naive (93%), compared to treatment experienced (77%) patients (326, 328, 331). Given these results, in 2015, first-line therapy for genotype 3 infection utilizing sofosbuvir includes the combination of ribavirin plus weekly peginterferon for 12 weeks, and second-line therapy for those who cannot tolerate interferon includes sofosbuvir and weight-based ribavirin for 24 weeks.

Daclatasvir and Sofosbuvir Combination Therapy

As mentioned above, the combination of daclatasvir with sofosbuvir was approved in 2015 by the FDA for therapy of HCV genotype 3 infection. One phase III study demonstrated a 97% SVR rate in treatment naive patients without cirrhosis, and a 58% SVR rate in those with cirrhosis (332). As such, this regimen has been administered for 12 weeks in those without cirrhosis and 24 weeks with or without weight-based ribavirin in those with cirrhosis (255).

HCV Genotype 4 Therapy

During the era of interferon-based therapies, treatment recommendations for genotype 4 were identical to that of genotype 1 as both were thought to be "difficult-to-treat" populations. Therefore, dual therapy with peginterferon and ribavirin was administered for 48 weeks. The combination of sofosbuvir with weekly peginterferon and weight-based ribavirin resulted in a 96% SVR rate when administered for 12 weeks (331). In the current DAA era, while there are no FDA approved therapies or phase III studies for genotype 4 infection, data suggest that three DAA combinations may be effective for those infected with this genotype.

Ledipasvir and Sofosbuvir Combination Therapy

In two small phase II studies, a 95 to 100% SVR rate was seen with the combination of ledipasvir and sofosbuvir for 12 weeks in treatment-naive and treatment-experienced patients regardless of fibrosis stage (333, 334). Therefore, therapy with this combination has been used off-label in genotype 4 patients for 12 weeks.

Ombitasvir, Dasabuvir, and Ritonavir-Boosted Paritaprevir Combination Therapy

The combination of ombitasvir (an NS5A inhibitor), dasabuvir, and ritonavir-boosted paritaprevir with or without ribavirin was evaluated in a small phase IIb study (335). This study evaluated this therapeutic regimen for 12 weeks in 86 treatment naive patients without cirrhosis and resulted in a 100% SVR rate in those that received weight-based ribavirin, and a 91% SVR rate in those that did not receive ribavirin. Given these results, this combination has been used off-label with weight-based ribavirin for 12 weeks in treatment-naive patients with genotype 4 infection.

Sofosbuvir with Weight-Based Ribavirin Combination Therapy

The combination of sofosbuvir with weight-based ribavirin (336, 337) given for 24 weeks appears to be better than 12 weeks of therapy, with SVR rates above 90%. However, in HIV/HCV co-infected patients, only 84% achieved an SVR (338). Thus, this combination has been administered for 24 weeks in treatment-naive patients infected with HCV genotype 4.

HCV Genotypes 5 and 6 Therapy

Historically, there has been a paucity of data in treating patients with HCV genotype 5 or 6 chronic infection, even during the era of combination therapy with peginterferon and ribavirin. Although these genotypes are seen worldwide they are seen in much lower frequencies than genotypes 1 to 4. During the early availability of sofosbuvir, only 7 patients (1 with genotype 5 and 6 with genotype 6) were treated with the combination of sofosbuvir plus peginterferon and weight-based ribavirin for 12 weeks, all of whom achieved an SVR (331).

Ledipasvir and sofosbuvir is the only interferon-free DAA combination that has been evaluated for genotype 5 or 6 infection. In two small open-label studies an SVR rate of approximately 96% has been demonstrated with this regimen when treated for 12 weeks (255, 339).

Future Therapeutic Development

Aside from the various therapeutic targets identified in the HCV replication cycle, including studies evaluating sofosbuvir and the new NS5a inhibitor velpatasvir, future potential targets of therapy include miR122, HCV p7, immune modulatory agents, and viral entry inhibitors. Micro RNA-122 (miR-122) is highly expressed in the liver and has been shown to be essential in HCV replication, and is therefore a potential therapeutic target (27, 340). HCV p7 is a membrane-associated ion channel protein that is essential for capsid assembly and envelopment (341). Current immunomodulatory drug targets under investigation for HCV include TRL-7 agonists, caspase inhibitors, PD-1 receptor antibody therapy, and others. In an ongoing multi-center phase 1 study targeting miR-122 in HCV infected patients, preliminary results of this target appear promising with pangenotypic activity (342).

Viral binding and entry into the target cell have been areas of active research. Through targeting these mechanisms, one can prevent the initial step of entry of viral genomic material that can persist in infected cells (343). Although the entry of HCV is complex, numerous host receptors and pathways have been identified (344). Current potential targets of entry factors include heparin sulfate proteoglycans, scavenger receptor class B type I (SR-BI), CD81, claudin-1 (CLDN1), occluding (OCLN), Neimann-Pick C1-like 1 (NPC1L1), transferrin receptor 1 (TfR1), epidermal growth factor receptor (EGFR), and ephrin receptor A1 (EphA2). Thus, given these vast targets, the development of HCV entry inhibitors as therapies is a burgeoning field that hopes to yield fruitful results.

Another potential avenue includes the discovery of novel inhibitors of other steps of HCV life cycle. With the recent significant improvements of high-throughput cell culture screening systems, large-scale screening of compound libraries has become feasible along with the identification of new potential therapies for HCV (345). One such recent discovery includes the identification of the first-generation antihistamine chlorcyclizine HCL (CCZ) as a potent in-

hibitor of HCV replication (346, 347). This over-the-counter compound has been described to have the ability to block HCV entry in addition to reducing HCV replication in animal models, while displaying synergy with sofosbuvir, boceprevir, and telaprevir. Thus, with further clinical development, this therapeutic modality may provide low-cost alternative for HCV eradication.

Contraindications and Side-Effects to Direct-Acting Antiviral Therapy Regimens

Compared to the first approved DAAs, the contraindications and side effects of current DAAs have significantly improved. Contraindications for currently available DAA therapies are few and include pregnancy, breast-feeding, allergy to the specific medication, and intake of other medications that would alter the drugs metabolism. Each DAA has its own unique profile of medications that cannot be co-administered. For example: (i) sofosbuvir should not be administered with P-glycoprotein inducers as this can alter drug concentrations; (ii) sofosbuvir with co-administration of amiodarone can result in symptomatic bradycardia; and (iii) ombitasvir, paritaprevir/ritonavir plus dasbuvir should not be co-administered with drugs that are dependent on CYP3A for clearance, are inducers of CYP3A and CYP2C8, or are strong inhibitors of CYP2C8 (349–352). Thus it is paramount for the prescriber to be aware of potential drug-drug interactions on a case-by-case basis. Side effects of current DAAs are minimal and also drug specific. Taken together, the tolerability of current regimens has vastly improved, which has resulted in improved accessibility to patients, especially for those that are intolerant to interferon-based therapies.

CONCLUSION

Since the early 1970s, another form of infectious viral hepatitis separate from the hepatitis A and B was known to exist. Today, this virus is known as hepatitis C, which remains the leading cause for liver transplantation. Since its initial cloning and early characterizations in 1989, significant advances in the understanding, characterization, and treatment of HCV have been realized (3). Today, compared to a decade ago, given the multitude of advancements in this field, the possibility of global eradication of HCV appears to be an attainable goal. However, much remains to be done to significantly impact global health with this devastating viral infectious disease.

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Alphaviruses

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The alphaviruses are principally mosquito-borne, positive-strand RNA viruses in the family *Togaviridae* that exhibit a broad range of pathogenicity in humans and animals (1, 2). Members of the genus are distributed worldwide in diverse ecological niches, where they are usually maintained in cycles between mosquitoes and birds or mammals. While human infections generally are incidental to the transmission cycles, in some instances human-mosquito-human cycles can maintain transmission and lead to large outbreaks and epidemics. Among the 24 alphaviruses listed in Table 1, 16 have been associated with human illness. Clinically, these manifest most commonly as polyarthralgia, often accompanied by fever and/or rash, or as central nervous system (CNS) infections. In addition to the alphaviruses circulating between mosquitoes and vertebrate hosts, a single example of an alphavirus, restricted to mosquitoes, has recently been described, Eilat virus (EILV) (3), and there are two known aquatic species, southern elephant seal virus (SESV) and salmon pancreatic disease virus (SPDV), that are likely to have lice as vectors (4, 5). This chapter reviews general aspects of the virology, pathogenesis, laboratory diagnosis, and prevention of the alphavirus infections, followed by more detailed discussion of those that cause human disease.

Alphavirus infections occur throughout the world, historically causing predominantly encephalitis in the Americas and polyarthralgic illness elsewhere (6). Disease due to the New World alphaviruses was first recognized when outbreaks of equine encephalitis occurred in Massachusetts in the early 19th century, followed by other outbreaks in the United States and South America (7). In 1930, Western equine encephalitis virus (WEEV) was the first alphavirus successfully cultured; eastern equine encephalitis virus (EEEV) and Venezuelan equine encephalitis virus (VEEV) were also isolated from horses later that decade. The earliest descriptions of human disease due to Old World alphaviruses were epidemics likely of chikungunya virus (CHIKV) illness occurring in India and Southeast Asia starting more than 200 years ago. Outbreaks of polyarthritis, probably due to Ross River virus (RRV), were described in eastern Australia and New Guinea in the late 19th and early 20th centuries (8). CHIKV, RRV, and Sindbis virus (SINV) were subsequently isolated from mosquitoes and patients in the 1950s and 1960s, followed by Barmah Forest virus (BFV) from mosquitoes in the 1970s, and from humans in the 1980s.

Alphavirus encephalitis has remained a rare disease of declining incidence in animals and humans, though the threat remains because these viruses continue to circulate. However, infection and human disease due to enzootic VEEV may be underestimated (9). In contrast, the alphaviruses causing arthritis continue to cause substantial human illness worldwide with regular epidemics, including massive CHIKV outbreaks that have recently spread to the Pacific nations, Europe, and the Americas (10).

VIROLOGY

Classification

The alphaviruses form a genus within the family *Togaviridae*, with similarities in genomic organization to rubella virus (genus *Rubivirus* in the *Togaviridae*) (Chapter 56). The alphaviruses are classified into at least 7 antigenic complexes (Table 1); this classification is generally supported by analyses of available genomic sequences (Fig. 1) (1, 2). The phylogenetic division into complexes of viruses related to EEEV, VEEV, SINV, and Semliki Forest virus (SFV) diverges from the antigenic classification in the placements of Middelburg virus (MIDV) and WEEV. Although WEEV is antigenically related to SINV, genetically it is a recombinant of an EEEV-like virus and an ancestral, perhaps extinct, SINV-like virus (11, 12). The two aquatic alphaviruses, salmon pancreatic disease virus (SPDV), which includes its subtype sleeping disease virus, and Southern elephant seal virus (SESV), differ from the other alphaviruses in being louse-borne rather than mosquito-borne. EILV is the first identified insect-specific alphavirus and is defective for replication in vertebrates, both at the entry and RNA replication stages (3).

Genotypes and Antigenic Groups

Alphaviruses are estimated to have evolved at a rate of approximately 10^{-4} substitutions/nucleotide/year, approximately 10- to 100-fold slower than that of most non-arthropod-borne RNA viruses, presumably reflecting constraints imposed by alternating-host (vertebrate and arthropod) replication cycles (5). Molecular taxonomic studies, tracing the movements of alphaviruses between continents, have established the recent divergence of several medically important alphaviruses. This includes the

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TABLE 1 Selected characteristics of alphaviruses[a]

Antigenic complex	Virus	Subtype(s)/ variety	Clinical syndrome	Transmission cycle	Geographic distribution
EEE	**EEEV**		Encephalitis	Mosquito-bird	United States, Canada, Caribbean
	Madariaga virus		Febrile illness, encephalitis	Mosquito-mammal/bird	Central and South America
VEE	**VEEV**	**IAB, IC, IE** (epizootic/ epidemic)	Febrile illness, encephalitis	Mosquito/equine	South and Central America
		ID, IE, IF (enzootic)	Febrile illness, encephalitis	Mosquito-rodent/bird	South and Central America
	Mosso das Pedras virus	IF	Asymptomatic	Unknown	Argentina
	EVERV	**II**	Encephalitis	Mosquito-rodent	Florida
	Mucambo virus	**IIIA**	Febrile illness, encephalitis	Mosquito-rodent/ marsupial	South America, Caribbean
	Tonate virus	**IIIB**	Febrile illness, encephalitis	Mosquito-rodent/ swallow bug-bird	South and Central America
	71D-1252 virus	IIIC		Unknown	Peru
	Pixuna virus	IV	Asymptomatic	Unknown	Argentina, Brazil
	Cabassou virus	V		Unknown	French Guiana
	Rio Negro virus (AG80-663)	VI	Febrile illness (dengue-like)	Unknown	Argentina
WEE	**WEEV**[b]		Encephalitis	Mosquito-bird/hare	North and South America
		Buggy Creek virus		Swallow bug-bird	North America
	SINV[c]		Febrile illness, rash, arthritis	Mosquito-bird	Europe, Asia, Australia, Africa
		Ockelbo	Febrile illness, rash, arthritis	Mosquito-bird	Europe
		Kyzylagach virus		Mosquito-bird	Azerbaijan, China
	Trocara virus			Unknown	Brazil
	Fort Morgan virus			Swallow bug-bird	Western United States
	Highlands J virus			Mosquito-bird	Eastern United States
	Aura virus			Mosquito-mammal	Brazil, Argentina
	Whataroa virus			Mosquito-bird	Australia, New Zealand, Oceania
Semliki Forest	**SFV**		Febrile illness, encephalitis	Mosquito-mammal/bird	Africa, Asia
		Me Tri Virus	Encephalitis	Mosquito-mammal/bird	Vietnam
	CHIKV		Febrile illness, rash, arthritis	Mosquito-primate/ other mammal; mosquito-human	Africa, Asia, Americas
	ONNV		Febrile illness, rash, arthritis	Mosquito-human epidemic cycle	Africa
	MAYV		Febrile illness, rash, arthritis	Mosquito-primate, other mammal/ marsupial/bird	Central and South America, Caribbean
	Una virus		Asymptomatic?	Mosquito-primates, birds?	South America
	RRV		Febrile illness, rash, arthritis	Mosquito-mammal; mosquito-human (epidemic)	Australia, Oceania

(*Continued*)

TABLE 1 Selected characteristics of alphaviruses[a] (*Continued*)

Antigenic complex	Virus	Subtype(s)/ variety	Clinical syndrome	Transmission cycle	Geographic distribution
	Getah virus		Fever?	Mosquito-pig/horses, other mammals	Asia, Australia
		Sagiyama virus		Mosquito-horses	Japan
	Bebaru virus			Mosquito-?	Malaysia
Barmah Forest	**BFV**		Febrile illness, rash, arthritis	Mosquito-mammal	Australia
Middelburg[e]	Middelburg virus			Mosquito-?	Africa
Ndumu	Ndumu virus			Mosquito-mammals?	Africa
Salmon pancreas disease	Salmon pancreas disease virus[f]				Aquatic
	Sleeping disease virus				Aquatic
Southern elephant seal	Southern elephant seal virus			Lepidophthirus macrorhini	Aquatic
Eilat	Eilat virus[g]			Mosquito host	Israel

[a]Viruses in bold type are human pathogens. Individual viruses are distinguished by standard serologic procedures; strains that exhibit slight differences (e.g., unidirectional 4-fold difference in cross-neutralization tests) are regarded as subtypes, and lesser but consistent antigenic differences define viral varieties.
[b]WEEV is a genetic recombinant of EEE-like and ancestral Sindbis-like viruses but is antigenically related to Sindbis complex viruses.
[c]Additional antigenic subtypes or varieties are recognized.
[d]SINV is the official type species of the Alphavirus genus.
[e]Middelburg virus is antigenically distinct from viruses in other alphavirus complexes but is related genetically to SFV.
[f]Salmon pancreas disease virus and Southern elephant seal virus are both aquatic viruses.
[g]Eilat virus is the first known mosquito virus, unable to replicate in vertebrate cells and adapted to a single mosquito host.

clustering of the New World MAYV with the Old World arboviruses (11) and the emergence of the Indian Ocean lineage of CHIKV in 2005 (10). The geographic origin of the alphaviruses is unclear, but recent phylogenetic analyses suggest a possible origin from louse-borne fish alphaviruses, SPDV being the only currently known representative, which then adapted to terrestrial mammals and mosquitoes (4). In that scenario, subsequent movement of viruses between the hemispheres and further recombination between Old and New World viruses contributed to the diversity (1, 2, 11). For example, a later recombination between ancestral EEEV-like and SINV-like viruses occurring more than a thousand years ago, presumably in the New World, generated a recombinant that subsequently diverged into WEEV and related Fort Morgan (FMV) and Highlands J (HJV) viruses (12). Where this recombination event occurred is unknown, but authors have variously suggested that it took place in the Old World (11) or in the New World and either in South America (12) or North America (13). However, the recent discovery of the insect-specific EILV may mean that the mosquito-borne alphaviruses descended from insect-specific viruses through adaptation to vertebrate hosts (14), either directly or via a fish-louse alphavirus.

EEEV, originally designated as a single species with four antigenic subtypes (I–IV), recently has been divided into EEEV (subtype I found in North America and the Caribbean) and Madariaga virus (MADV) (subtypes II–IV found in South America) (15). Both EEEV and the MADV subtypes are associated with equine disease. North American EEEV (subtype I) has been associated with severe human disease for decades, but only in 2010 was MADV associated with an outbreak of human encephalitis in Panama (16). The North American lineage is highly conserved both genetically and antigenically, while MADV is more diverse. The lack of virulence of most MADV strains in humans may be related to their greater sensitivity to human type I interferons (17).

The VEEV antigenic complex, a sister group to EEEV, is even more diverse, with at least 10 different subtypes and varieties. These viruses are distributed in a mostly nonoverlapping pattern in the Central and South American tropics and subtropics. The greater diversity of the VEEV complex may be explained by the low mobility of their rodent hosts compared to the avian hosts of EEEV. WEEV, which also cycles among birds, also exhibits highly conserved lineages spanning North and South America (18, 19).

Composition

The alphaviruses are small icosahedral viruses with a lipid-bilayer envelope closely enshrouding a nucleocapsid with a diameter of 40 nm (Fig. 2) (2). Eighty glycoprotein spikes extend from the virion surface in a T=4 lattice, giving the virion a total diameter of 70 nm. The flower-like spikes, trimers of E1 and E2 heterodimers, are anchored in, and span, the envelope to form 1:1 associations with nucleocapsid monomers via the carboxy-terminal E2-specific peptide. The positively charged N-terminal domain of capsid protein mediates specific packaging of alphavirus genomes into nucleocapsid, and the interactions of C-terminal domains determine the icosahedral T=4 symmetry of the nucleocapsid. Interaction of the latter domain with glycoprotein spikes also drives the virion budding process.

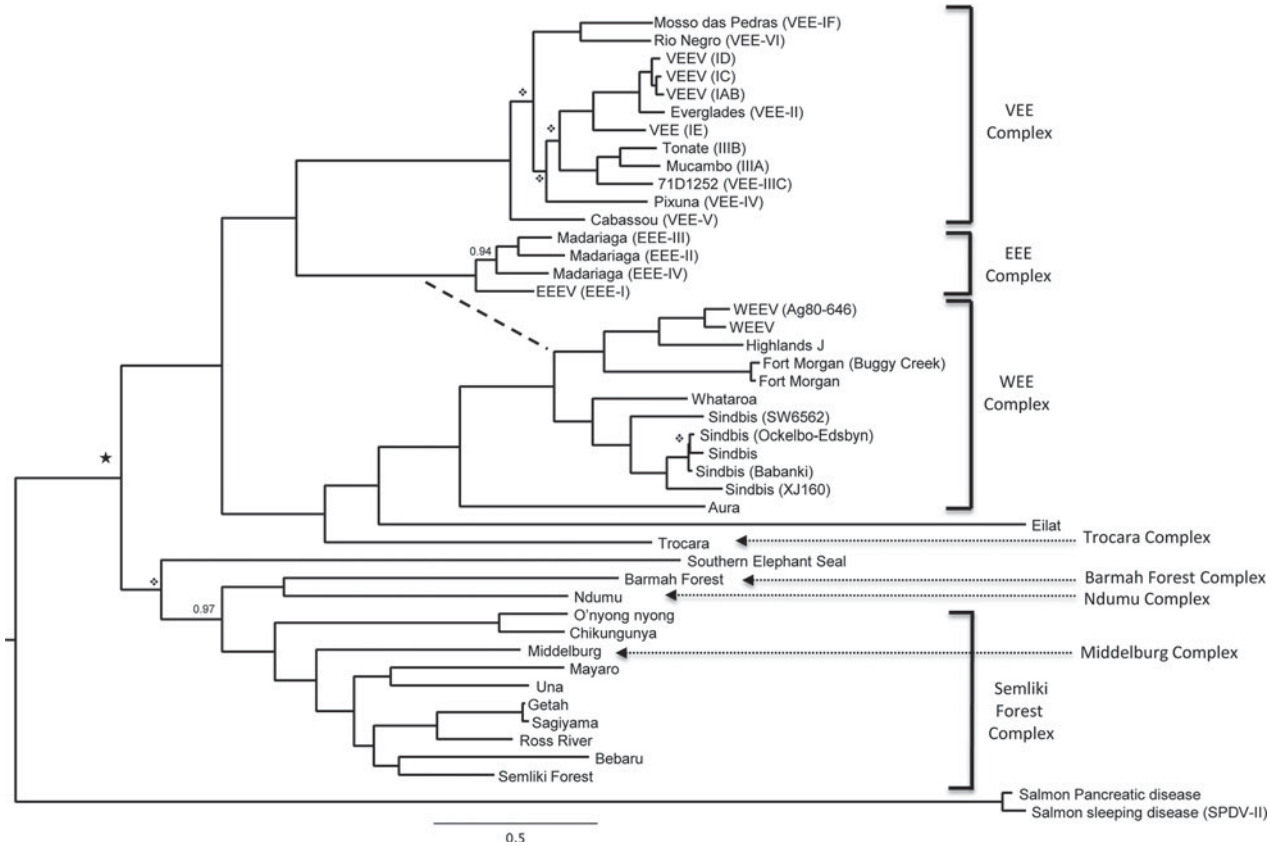

FIGURE 1 Phylogenetic tree of the alphavirus species and selected variants based on the structural protein (E2, 6K, E1) amino acid sequences constructed using Bayesian methods and midpoint rooting. Terminal nodes are labeled by virus species, subtype, or variant, in parentheses. The dashed line indicates the point at which ancestors of Sindbis and Madariaga viruses recombined to form the recombinant WEEV group. All posterior probabilities were 1 except nodes with a ❖ symbol had posterior probabilities less than 0.9 and nodes with a ★ had no posterior support. Adapted from reference 186 with permission.

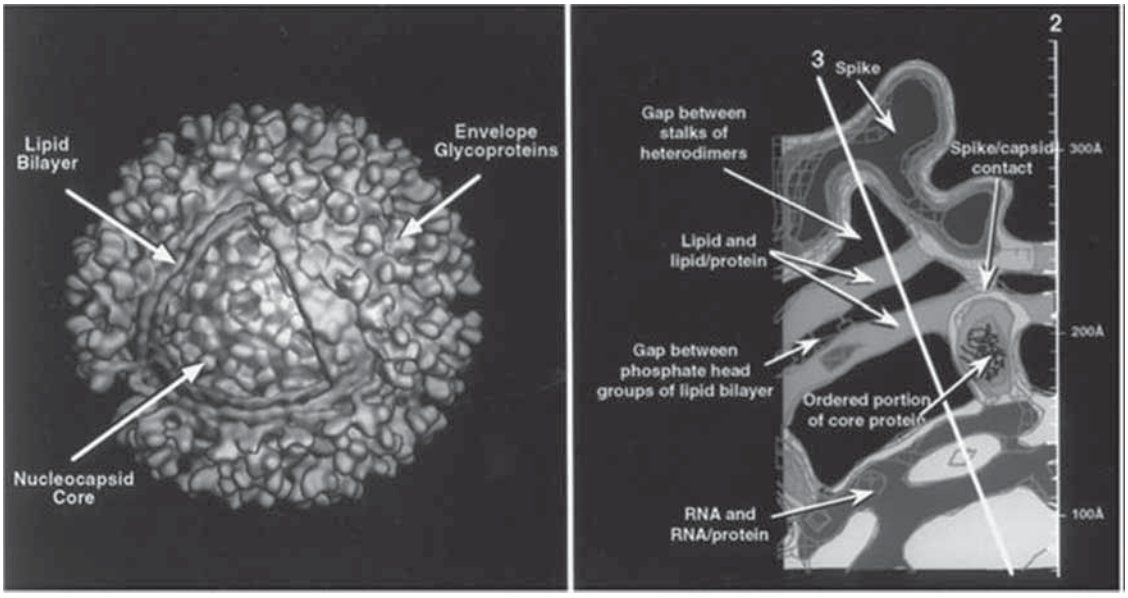

FIGURE 2 Ross River virus (RRV): (Left) Cryoelectron microscopic image reconstruction of RRV showing the flower-like envelope protein spikes, virion membrane, and nucleocapsid core. (Right) Relationships of spike and capsid proteins and virion RNA. Courtesy of R. J. Kuhn.

The viral genome is a single strand of positive-sense RNA, 11 to 12 kb in length, with a poly(A) tail and a 5′-terminal cap (Fig. 3). The genome consists of an open-reading frame (ORF) that occupies the 5′ two-thirds of the genome and encodes the four nonstructural proteins, while the 3′ one-third has an ORF encoding the three structural proteins (1). The nonstructural proteins are translated as a polyprotein that subsequently is cleaved into a number of functional intermediates and ultimately to four nonstructural proteins (nsP1–4) that are required for replication. The structural proteins are translated from a subgenomic mRNA (approximately 4.1 kb) and cleaved from a polyprotein into two major-envelope proteins (E1, E2), the capsid protein, the minor envelope protein E3, and the transmembrane 6K/TF proteins. Conserved sequences in the junction between nonstructural and structural domains and in 5′- and 3′-terminal untranslated regions serve as promoters in RNA replication and transcription of the subgenomic RNA.

Characteristics of structural and nonstructural proteins derived from crystal structures (20, 21) and cryoelectron microscopic reconstructions (22), are summarized in Table 2. Multiple domains on the E2 protein are principally associated with antibody-mediated viral neutralization of which the most important is a small linear sequence of two antigenic sites between amino acids 180 to 216 for SINV and VEEV and in a corresponding position for RRV; other domains are discontinuous and conformationally dependent. The E1 glycoprotein contains the viral hemagglutinin, fusion properties, and cross-reactive neutralizing epitopes. Epitopes that elicit protective nonneutralizing antibodies have been identified.

Studies conducted using SINV-infected mice as an encephalitis model have linked neurovirulence to specific E1 and E2 amino acid substitutions. In particular, a change from glutamine to histidine at position 55 of the E2 protein induces neuronal apoptosis by blocking apoptotic inhibitors such as *bcl-2*; a similar effect on apoptosis can be seen *in vitro* in mouse neuronal cells and the human cancer cell line AT-3 (23, 24). Attenuating loci have been identified at other E2 loci, in the 5′ noncoding region, and in nsP1 of SFV, VEEV, and SINV. Natural viral determinants of equine virulence, associated with epizootic VEEV emergence and viral capacity to replicate and disseminate in mosquito vectors, also have been identified in the E2 protein (18).

Replication

Several different cellular protein receptors in vertebrate and mosquito cells have been identified for alphaviruses. The high-affinity laminin receptor serves as a mammalian and mosquito cell (*in vitro*) receptor for SINV, whereas other protein receptors for SINV have been identified in mouse neural and chicken cells. Collagen-binding α1β1 integrin has been identified as the RRV receptor on fibroblasts (25). A number of other potential CHIKV receptors have been identified, but their roles remain unclear (26). Glycosaminoglycans have been implicated as mammalian cellular receptors or enhancers of viral binding for SINV, SFV, CHIKV, and RRV (26, 27). Other less-conserved protein receptors or accessory factors may be involved in infection of the mosquito midgut. After binding to the cell membrane, virions are taken up in clathrin-coated endocytic vesicles (Fig. 4). Virion spikes, especially through the E1

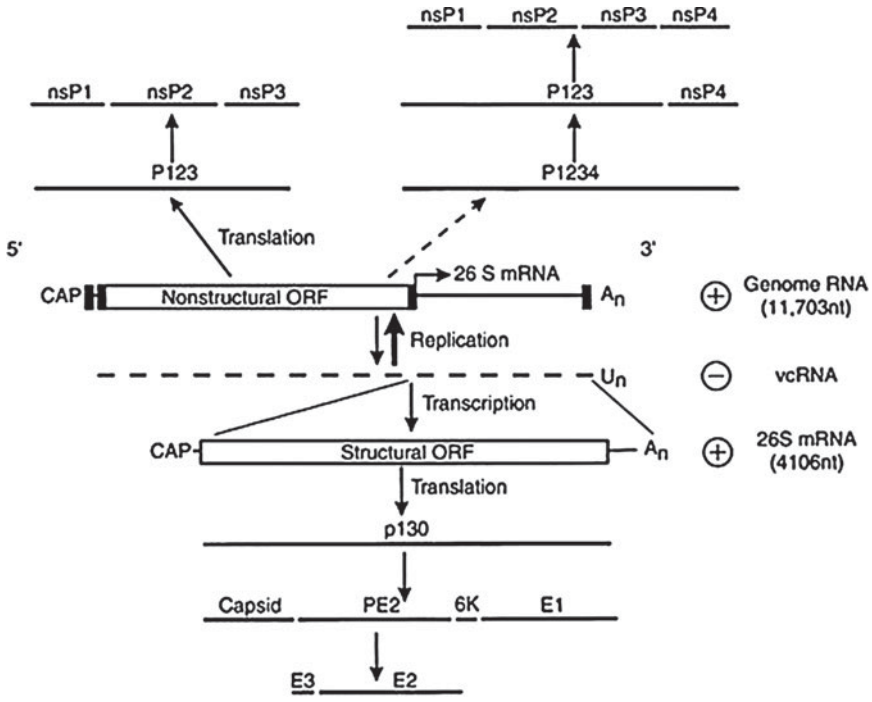

FIGURE 3 Schematic diagram of alphavirus genome [positive (+) sense RNA with poly(A) (A_n) tail and 5′ terminal cap (CAP); viral complementary RNA (vcRNA) of negative (-) sense; and subgenomic mRNA of positive sense]. Viral nonstructural proteins are translated from the 5′ two-thirds region of the genome, yielding polyprotein intermediates and nonstructural proteins nsP1 to nsP4. RNA is replicated into negative-sense RNA templates and transcribed into 26S subgenomic RNA. Cotranslational processing of the subgenomic mRNA yields the three principal structural proteins: capsid and envelope glycoproteins (E1 and E2). ORF, open reading frame; nt, nucleotide.

TABLE 2 Alphavirus proteins

Protein	Length (amino acids)[a]	Characteristics and function
Structural		
Capsid	264	30 kDa monomers organized as 12 pentamers and 30 hexamers; multiple copies bind to viral RNA genome, ribosome, and cytoplasmic domain of transmembrane spike
E1	439	52 kDa glycosylated transmembrane (spike) protein; fusion domain for viral membrane penetration; hemagglutination, neutralization, and protection
E2	423	49 kDa glycosylated transmembrane (spike) protein; epitopes for viral neutralization, neurovirulence, hemagglutination, viral receptor interactions
E3	64	10 kDa glycoprotein cleaved with E2 from PE2 intermediate; associated with virion spike in SFV
Nonstructural		
nsP1	540	Initiation of RNA minus-strand synthesis; guanyltransferase function, modulation of nsP2 activity
nsP2	807	N-terminal domain—RNA helicase, NTP binding, initiation of 26 mRNA transcription; C-terminal domain—nonstructural proteinase; 50% of SFV activity in nucleus (function unknown)
nsP3	556	N-terminal domain conserved in length and sequence; C-terminal domain variable in length and sequence; functions unclear
nsP4	610	RNA polymerase; unstable and rapidly degraded

[a]Length in SINV.

fusion-promoting protein, induce bridging of virion and vesicle membranes with pH-dependent release of the nucleocapsid into the cytoplasm. The acidic conditions in the vesicle lead to shrinkage of the capsid, exposure of a ribosomal binding domain, and uncoating, with release of RNA from the capsid matrix. In the cytoplasm, the positive-sense viral genome functions as mRNA. Nonstructural proteins are translated as polyproteins, principally as a P123 moiety when translation is terminated by an opal (UAG) codon near the 3′ end of the nsP3 gene, or when replaced by a sense codon or through translational read-through as a P1234 polyprotein. Replicase activities are associated with the polyprotein intermediates and individual nonstructural proteins, as well as host factors (1). Sequential processing of nonstructural polyprotein differentially regulates synthesis of alphavirus-specific RNA species at different replication steps.

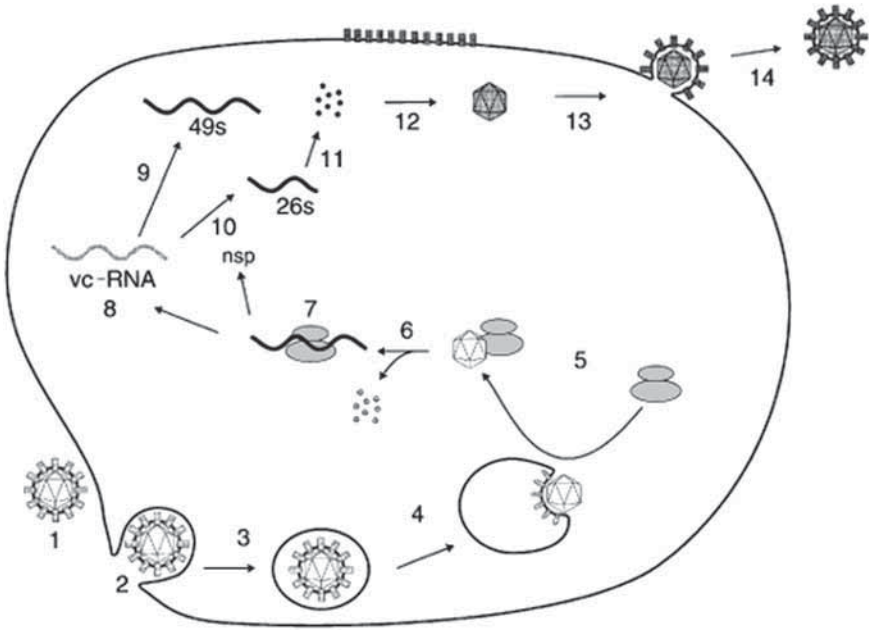

FIGURE 4 Replication of alphaviruses. After binding to the cell membrane (step 1), virions are taken up in endocytic vesicles (steps 2 and 3). The virion and vesicular membranes fuse, releasing the nucleocapsid (step 4). The viral nucleocapsid binds to a ribosome (step 5) and is uncoated, freeing viral RNA (step 6) from the individual capsid (gray dots). Positive-sense viral RNA (heavy line) is replicated (step 7), producing complementary negative-sense RNA (gray line) (step 8), which, in turn, is transcribed to full-length genomic positive-sense RNA (step 9) or subgenomic RNA (step 10). Nonstructural proteins (nsp) are translated from genomic RNA. Subgenomic RNA is translated to produce capsid proteins (black dots) and envelope proteins, which are modified before insertion into the cell membrane (gray bars) (step 11). Genomic RNA is packaged with capsid proteins into nucleocapsid cores (step 12); capsid and E2 proteins associate (step 13) prior to viral budding (step 14).

The replication complexes of alphaviruses are initially associated with the plasma membrane, after which most are transported into the cytoplasm on the membranes of endocytic vesicles. Association with cytoplasmic membranes is achieved by a specific peptide in nsP1, which is also modified by palmitoylation of cysteine residues. RNA is replicated with the production of negative-sense RNA templates, which are present in the cells as dsRNA duplex with positive-strand RNA. They are later transcribed either to full-length genomic RNA or, when initiated at the internal junction site, to subgenomic RNA. The 5′ ends of both genomic and subgenomic RNA are capped with Cap(0), which makes alphavirus RNAs different from cellular mRNAs, because the first nucleotide downstream of Cap itself is not methylated. Genomic alphavirus RNA can bind to the viral replicase, producing more negative-strand templates, which then bind to ribosomes and is translated into additional nonstructural proteins. These non-structural proteins also lead to the transcription of the subgenomic 26S mRNA, which is then cotranslated to form the structural polyprotein. A rapid autoproteolytic process then cleaves the capsid protein from the 5′ end of the polyprotein. This cleavage exposes an N-terminal signal sequence that facilitates transport and insertion of the PE2 polyprotein (containing E2) into the endoplasmic reticulum (ER) membrane. Subsequent signals on a structural polyprotein intermediate lead to insertion of the 6K protein and translocation of E1. Ultimately, the structural proteins are processed into capsid, PE2, and E1. The latter envelope proteins are modified in the ER lumen by addition of carbohydrate chains and fatty acids and are further modified during transport through the Golgi apparatus. PE2-E1 dimers are formed in the ER; during transport through the Golgi apparatus, PE2 is cleaved to form E2 and E3. The envelope spikes are transported to the cell plasma membrane, where the E2 cytoplasmic domain associates with the nucleocapsid C-terminal domain prior to viral budding.

Besides being the components of the viral replication machinery and of the virions, some alphavirus-specific proteins exhibit other critical functions in virus replication. The capsid protein of the New World alphaviruses, like EEEV and VEEV, inhibits nucleocytoplasmic traffic (28, 29), and the nsP2 protein of the Old World alphaviruses, like SINV, SFV, and CHIKV, mediates degradation of the main subunit of cellular DNA-dependent RNA polymerase (17). These two different mechanisms lead to similar results: rapid inhibition of transcription in vertebrate but not in insect cells (30), and thus downregulation of the innate immune response in the former.

The highly productive and rapid alphavirus replication cycle (see below) has been exploited in a novel approach to produce nonreplicating particles (replicons) as immunogens. Foreign genes from a variety of viruses, including human immunodeficiency virus, Ebola virus, and Marburg virus, have been introduced in place of VEEV or SINV structural protein genes, with helper systems providing the alphavirus structural proteins that allow packaging of recombinant particles that cannot replicate, while allowing high-level expression of the desired antigen.

Host Range in Animals

Alphaviruses are transmitted to vertebrate hosts principally by mosquitoes but in some instances by ticks, mites, or other arthropods. The capacity of specific mosquito vectors to transmit infection requires competency to support viral replication in their midgut cells, followed by dissemination within the mosquito and infection of salivary glands (extrinsic incubation period). Salivary gland infection and the resultant capacity to transmit infection with subsequent blood feedings often persist for the life of the mosquito. Barriers to midgut and salivary gland infection have been identified in some species; e.g., a midgut barrier to CHIKV infection in *Aedes* (*Stegomyia*) *aegypti* has been linked to eye color at a chromosome 3 locus. Recently, several point mutations in the CHIKV Indian Ocean Lineage (IOL), that initially emerged from Kenya in 2004, have been identified that confer increased infectivity for the midgut of *A.* (*Stegomyia*) *albopictus* (31–33). These same mutations have little or no impact on infection of *A. aegypti*, and the critical E1-A226V substitution has no impact in Asian Lineage strains because they have an epistatic constraint that restricts their ability to adapt to this new vector (34). Transovarial transmission of RRV in mosquitoes has been demonstrated experimentally and confirmed in Australian field studies.

Natural alphavirus infections are observed in a broad range of animal species, including birds, mammals, reptiles, and amphibians. Only animals that develop a higher-titer viremia (approximately. $\geq 10^{3.5}$/ml) for a sustained period of several days contribute to further virus transmission. Many alphaviruses cause clinically silent infections of birds and rodents. Individual alphaviruses produce symptomatic infections in various animal species as described below. However, horses are unusually susceptible to a number of alphaviruses, e.g., EEEV, WEEV, VEEV, SFV, Getah virus (GETV), Una virus (UNAV), RRV, and Middelburg virus, while EEEV is virulent for an extensive range of avian and mammalian species.

Alphaviruses produce lethal infections in newly hatched chicks and embryonated eggs. Mice exhibit an age-dependent susceptibility; 2- to 3-day-old suckling mice develop fatal encephalitis when inoculated intracerebrally, while weanling and older mice are variably susceptible after intracerebral or peripheral inoculation. Differential ratios of fatal infection after inoculation by these routes have been linked to neuroinvasiveness and virulence of specific viral strains. Some strains of o'nyong-nyong virus (ONNV) kill suckling mice only after adaptation by serial passage. Other laboratory animals, such as hamsters, guinea pigs, rats, rabbits, and nonhuman primates, develop encephalitis after intracerebral inoculation with encephalitogenic alphaviruses. Animal studies on joint and bone disease due to SINV, CHIKV, RRV, and BFV have been conducted in mice, as discussed under the individual viruses below.

Growth in Cell Culture

Alphaviruses can be propagated in a variety of cell cultures but generally grow best in mosquito cell lines, such as C6/36, AP-61, and TRA-284. As may be expected with viruses that replicate in insects at ambient temperatures, they often grow better at low temperatures. However, the cytopathic effect (CPE) is minimal or absent in mosquito cells, so blind passage to another cell line is required. Infection rapidly causes destructive CPE in primary duck and chicken embryo cells and in most mammalian cell lines, including BHK-21, Vero, and HeLa cells. EEEV and VEEV produce cell rounding, degeneration, and lysis within 18 to 24 hours and complete destruction of cell monolayers by 48 to 72 hours; WEEV-induced CPE appears 24 to 48 hours later. Viral yields usually range in the order of 10^7 to 10^{10} plaque-forming units (PFU)/ml. These viruses are adapted to mosquitoes and to birds, whose body temperatures can exceed 40°C, and viral

replication *in vitro* occurs over a broad range of temperatures, from 25 to 41°C. In mosquito-cell cultures, after an initial phase that may produce some degree of CPE, a persistent infection without visible cytopathology can be maintained indefinitely. Numerous differences in viral nucleotide use, maturation, and replication have been demonstrated in mammalian and insect cells (5).

Inactivation by Physical and Chemical Agents

As enveloped RNA viruses, alphaviruses are labile in the environment and are inactivated by lipid solvents like ether and chloroform, by deoxycholate, and by common laboratory disinfectants, such as hypochlorite, 70% alcohol, phenol, paraformaldehyde, and formaldehyde. Commercial procedures to eliminate potential viral contaminants of human biologics are effective in removing alphaviruses, e.g., ethanol fractionation and polyethylene glycol precipitation, virucidal treatments with tri(*n*-butyl) phosphate in combination with sodium cholate or Tween, and pasteurization at 60°C for 1 hour (35).

PATHOGENESIS

Natural infection of vertebrates is initiated when mosquitoes deposit infected saliva in extravascular tissues while blood feeding. Most alphaviruses replicate initially in skeletal muscle cells in mouse models (36), although VEEV is taken up into Langerhans cells. The latter may be important in natural human infections, with virus then travelling within leukocytes to draining lymph nodes and eventually spreading to target tissues in the nervous system, joints, or skin.

Neurotropic Alphaviruses

For the neurotropic alphaviruses, local replication results in a brief viremia, followed by CNS invasion through the cerebral vascular endothelium or olfactory epithelium. Like the arthropod-borne flaviviruses, the pathology typically includes the central cerebral structures (substantia nigra, thalamus, brainstem) and the spinal cord. Unlike EEEV and WEEV, which are principally neurotropic in humans, VEEV also produces a systemic illness in humans with pathological changes in the lung and lymphoid tissue of the gastrointestinal tract, spleen, and peripheral nodes (37). CNS pathology in VEEV infection reflects both viral and immunopathological processes, in which the induction of cytokines, interferons, TNF-α, and NO appear to be important in the balance of viral clearance and immune-mediated damage.

Variants of VEEV produced by site-directed mutagenesis have elucidated patterns of viral spread in mice after peripheral inoculation; specific changes in the E2 protein have been associated with delayed and sporadic infection of local lymph nodes, limitation of viral spread beyond the lymph nodes, and infection of local lymph nodes and other lymphatic tissue without significant viremia or neuroinvasion. Comparing the neurotropic wild-type VEEV Trinidad donkey IAB strain with its attenuated (vaccine) derivative (TC-83), in mice, attenuation appears to involve a combination of alterations in the E2 glycoprotein, perhaps affecting viral attachment and cell entry and, in the 5′-untranslated region, affecting viral RNA synthesis. A mutation at amino acid position 120 in E2 corresponds to a locus associated with attenuation of SINV strains exhibiting rapid cellular penetration. The attenuated strain is more rapidly cleared from blood, reducing opportunities for neuroinvasion (faster cell penetration expedites peripheral viral clearance). The mutation in the 5′-noncoding region may reduce viral replication in infected CNS cells, leading to decreased necrosis, inflammation, and CNS spread. The vaccine's limited neurovirulence has been shown by direct intracerebral inoculation of horses, which results in a minimal inflammatory reaction. Thus, attenuation is speculated to be a combined effect of reduced neuroinvasion and neurovirulence. In contrast to VEEV, EEEV induces a lesser interferon response in human myeloid-lineage cells and appears to evade host defenses via sites in the 3′-UTR that bind to microRNAs (38). Studies using a chimeric EILV/VEEV virus in vertebrate cells found that the IFN-β sensitivity of several of the alphaviruses, including EEV, VEEV, and CHIKV, appears to be due to inhibition of translation, resulting from activation of interferon stimulated genes including IFIT-1, which binds to the 5′-noncoding region (39). Mutations that alter the RNA secondary structural motifs within that region confer resistance to IFIT-1 in mice (40).

In humans, an age-dependent susceptibility of infants and the elderly to CNS infection has been observed epidemiologically, although its pathogenesis has not been elucidated.

The mechanism of antibody-mediated protection against alphavirus infection is only partially understood. Clearance of SINV from the murine nervous system in nonfatal infections relies on antibodies to the E2 glycoprotein and does not kill infected neurons. Release of budding virus is prevented and viral replication is inhibited through unknown antiviral mechanisms not requiring an interferon response; however, this nonlytic mechanism that controls viral infection results in viral RNA persistence (41). IFN-α/β protects adult mice from SINV infection by rapidly conferring an antiviral state on otherwise permissive cell types, both locally and systemically.

Arthritogenic Alphaviruses

In alphavirus infections characterized by rash and polyarthritis, the virus infects human monocyte/macrophages and dendritic cells, fibroblasts of the skin and synovium, muscle cells, and possibly keratinocytes, and it has also been found in periosteum in mice (42, 43) inducing an inflammatory response. Infection of skin structures in humans has been demonstrated by viral isolation and direct detection by PCR for SINV and by immunohistochemical staining of RRV antigen in basal epidermal and eccrine duct epithelial cells (44, 45). Skin biopsy samples from RRV patients show a perivascular mononuclear cell infiltrate of predominantly cytotoxic T cells, which, in the rare cases of purpuric rash, also lead to capillary damage and extravasation of blood (44). In experimentally infected mice, viral infection of, and extensive necrotic changes in, muscles, tendons, connective tissue, and periosteum offer a possible explanation for the musculoskeletal symptoms in humans, probably mediated by the inflammatory response (42). Macrophages have been directly implicated as the primary mediators of tissue damage. RRV infects macrophages both via a natural virus receptor and by Fc receptor–mediated antibody-dependent enhancement. Infection interferes with transcription of the interferon regulatory factor 1 and NF-*k*B genes and with translation of tumor necrosis factor and inducible nitric oxide synthase.

Joint fluids from the acute and chronic phases of human RRV disease contain an exudate comprised almost entirely of mononuclear cells that exhibit vacuolation, mitotic figures, and other signs of activation. Synovium shows an extensive mononuclear cell infiltrate, predominantly cytotoxic T cells, with areas of necrosis and fibrin deposition (46, 47). Viral antigen and RNA, but not infectious virus, have been

detected in joint synovium and macrophages for as long as 5 weeks after onset of symptoms (43). RRV, Barmah Forest virus (BFV), CHIKV, and SINV infect synovial monocytes/macrophages in animal models and some *in vitro* systems, whereas RRV can also be found in synovial cells of mice (47). A combination of release of inflammatory mediators from the infected monocytes/macrophages and the cytotoxic T-cell responses to viral antigens is the likely explanation for the synovial swelling, effusion, and joint pain experienced in acute human alphavirus infection.

Genetic susceptibility to RRV polyarthritis in humans has been linked to Gm phenotype and HLA-DR7 haplotype, which may have a role in reducing the cytotoxic T-cell response and delaying viral clearance, while SINV disease is associated with the DRB1*01 allele (47–49).

Joint symptoms persist for months or years in a substantial proportion of patients with alphavirus polyarthritis. This is likely caused by ongoing immune stimulation due to persistence of virus or viral components (42, 43), rather than being an autoimmune disease, though SINV arthritis in Finland has been associated with increased frequency of autoantibodies, including rheumatoid factor (49). RRV RNA has been shown by RT-PCR to persist within synovial tissue, and the virus can also persist in macrophages *in vitro* and *in vivo*, and residual CHIKV has been found up to 18 months postinfection, even in the presence of neutralizing antibodies, activated T cells, IFN-α, and IFN-β (43, 50). Alphaviruses have a range of possible strategies to avoid host immune cells, including chronic replicative infection of macrophages, reducing recognition of infected cells by cytotoxic T cells due to reduced MHC expression on the cell surface, reducing the susceptibility of infected cells to IFN-α and IFN-β, and possibly even integrating into host cell DNA. This appears to be accompanied by virus-induced impaired-host responses, such as a reduction of IFN responsiveness and macrophage antiviral responses (43). The chronic phase of joint disease is characterized by a monocyte/macrophage/NK cell and CD-4 T-cell response in the synovial fluid and elevated levels of a number of inflammatory mediators, including IL-6, IL-1beta, IFN gamma, CCL-2, and TNF. Although there is no evidence that alphavirus infections trigger autoimmune diseases, such as rheumatoid arthritis (RA), the inflammatory responses resemble those seen in RA. Mouse models have shown that infection with CHIKV and RRV triggers osteoclastogenesis resulting in both local and systemic bone loss mimicking that seen in RA (42). Studies conducted in humans also suggest a pathogenic inflammatory processes, as chronic joint disease following RRV or SINV infection is more common in patients who have pre-existing degenerative joint disease that contributes to the inflammation (42, 43).

The pathogenesis of severe CHIKV disease remains unclear (10) but presumably relates to the same pro-inflammatory responses associated with milder disease and possibly to poor IFN-α/β responses. The cause of the hemorrhagic diathesis in some cases of chikungunya is uncertain.

LABORATORY DIAGNOSIS

Viral Detection

Most alphaviruses are assigned to BSL-2 level (51). However, the encephalitic alphaviruses (EEEV, WEEV, VEEV, MADV, Cabassou, Mucambo, and Tonate viruses) are classed as BSL-3 agents due to the severity of illness, known respiratory transmission, and demonstrated laboratory-acquired infections. Similarly CHIKV and SFV are also currently classified as BSL-3 because a number of laboratory-acquired percutaneous and nonpercutaneous infections with CHIKV have been reported, as has a single case of fatal laboratory-acquired SFV (52). Where appropriate, and if available, staff working with live viruses should be immunized with VEEV and/or CHIKV vaccine.

The viruses can be isolated from blood, CSF, pharyngeal secretions, skin, and other tissue specimens, the most appropriate sources varying individually for each of the viruses. Most are readily isolated in a variety of cell lines found in diagnostic laboratories, including Vero, A549, and MRC-5 cells. Reference laboratories often employ mosquito cell lines and occasionally intrathoracic inoculation of mosquitoes. Spin inoculation has been shown to improve recovery of EEEV.

Antigen detection and PCR assays to detect viral products in blood, CSF, joint fluid, and skin have been described for several alphavirus infections, but few have been extensively evaluated. RT-PCR is more sensitive than culture, and, in CHIKV infection, detection of viral RNA in blood has been shown to be reliably positive in the first few days of illness, even in the presence of detectable antibody (10), unlike virus isolation. However, for most alphavirus infections, RT-PCR is usually negative by the time patients present to a medical practitioner and that, combined with the limited availability, means it is not currently used in routine diagnosis.

RT-PCR is superior to culture for detection of alphaviruses in trapped mosquitoes, including detection in expectorated saliva following feeding on sugar-impregnated nucleic acid preservation cards (53). Nucleic acid detection methods also assist molecular epidemiology and pathogenesis studies.

Serology

The most sensitive serologic assays detect virus-specific IgM by capture EIA or indirect IF methods. Specific IgM can be detected in serum within the first 7 to 10 days of illness in nearly all cases of alphavirus infection and in the CSF in encephalitis cases. However, serum IgM may persist for several months after acute infection and is not necessarily indicative of recent infection, and false positive IgM results have been problematic in some EIA tests (54, 55). Therefore confirmation by demonstrating rising IgG titers or seroconversion by HI, indirect IF, EIA, complement fixation (CF), or neutralization is recommended, particularly where there is not a clear clinical and exposure history. Detection of specific IgM in the CSF is considered diagnostic of recent encephalitis. IgM testing for alphaviruses is relatively specific, with cross-reactions usually occurring only among viruses within the same antigenic complex, e.g., among viruses related to SINV.

Cross-reactions are uncommon for all of these assays but are most likely with IF, EIA, and HI, whereas CF antibodies are relatively specific, and neutralization is highly specific. HI, IF, EIA, and neutralization antibodies rise in the first week after onset of illness, with a ≥4-fold change usually noted by the second week. HI and neutralization antibodies decline minimally after 30 months and may persist for years. CF antibodies rise more slowly and may not be detected until 2 to 3 weeks after illness onset. The half-life of CF antibodies is 2 to 3 years, and they remain detectable in only 15% of patients after 5 years, providing an alternate approach to identify recent past infections.

PREVENTION

General

Alphaviruses principally are maintained in zoonotic transmission cycles in natural habitats. Consequently, little can be done in an ecologically acceptable manner to control levels of virus circulation, and eradication is not feasible. At a societal level, consideration should be given to the environmental impact of human developments, such as water catchment areas, in increasing breeding of vector species and control of housing development near known mosquito breeding areas, which have been shown to correlate directly with RRV risk (56). However, prevention is primarily based on individual protection and public health measures to reduce vector numbers.

Approaches to vector control are tailored to specific viral transmission cycles and habits of individual vector species. In general, these can be divided into steps to eliminate sources of mosquito vectors by environmental modifications, to minimize the emergent vector mosquito population by applications of larvicides, and to reduce adult mosquitoes by the emergency application of adulticides using backpack sprayers, trucks, or planes.

Examples of source-reduction strategies include community projects to eliminate *A. aegypti* breeding sites in peridomestic containers to control CHIKV and environmental modifications, such as draining swamps and improving groundwater runoff, to control EEEV, WEEV, and RRV. Large-scale environmental modifications may be prohibitively expensive or conflict with other environmental priorities. Nevertheless, environmental modifications can achieve some reduction in vectors, especially when combined with systematic applications of larvicides to breeding sites.

Emergency vector control with adulticides can temporarily reduce vector mosquitoes that pose an immediate human risk. Typically, the decision to implement a large-scale adulticide program is stimulated by surveillance indicating large vector populations, high vector infection rates, seroconversions in sentinel animals, or cases in indicator animals, such as horses. These interventions are immediately effective in reducing adult mosquitoes on the wing, but infiltration of mosquitoes from surrounding untreated areas and their continued emergence necessitate repeated applications. The effectiveness of emergency vector control in preventing human disease has been difficult to prove because of inherent difficulties in conducting controlled evaluations under natural conditions. Large-scale insecticide applications are expensive and sometimes are met with local opposition because of concerns about pesticide toxicity for humans, birds, fish, and commercial bees. Nevertheless, adulticide use combined with public health advisories to avoid activities associated with exposure to vectors is the only available intervention to prevent epidemic transmission, and its expense, compared with the potential costs of even a single human EEE case, has been shown to be justified.

Recently there have been interesting developments of biological control measures, and particular success has been achieved with the use of modified endosymbiont bacterium (Wolbachia) to create noncompetent *A. aegypti* that displaces the natural population and interrupts dengue transmission. A number of other applications to other species and other viruses, including CHIKV and RRV, are being evaluated (57).

Personal Protection

Avoidance of mosquito exposure is the principal means of personal protection, including covering up exposed skin and using bed nets or sleeping in mosquito-screened or air-conditioned accommodation. Different species have different peak biting periods and preferred locations. For example, both *A. aegypti*, the major vector species for CHIKV and dengue, and *A. albopictus*, the alternative vector for these viruses, preferentially feed during the day, with peak periods in the early morning and late afternoon. So bed nets are most important if sleeping during the day. *A. vigilax*, which carries RRV, will bite at any time of the day or night, and bed nets are recommended at all times. In most risk areas for mosquito-borne diseases there are a range of species, habitats, and feeding patterns, meaning that it is prudent to take precautions at all times.

Mosquito repellents are an important part of personal protection. Diethyl-m-toluamide (DEET) is the most effective one that is approved for use on skin (58) and has a long track record. It has dual action in blocking the ability of the mosquito to locate hosts from a distance, as well as repellency on contact. It is available in concentrations up to 100%, but recommendations are that concentrations of 30 to 50% be used in adults and 30% in children older than 2 months. The higher the concentration of DEET, the longer lasting the protection. Ingestion and topical application have been associated with potentially fatal seizures and encephalopathy, principally in children. More than half of a topically applied dose penetrates the skin, and 17% is absorbed systemically. DEET toxicity should be included in the differential diagnosis of neurologic infections in patients exposed to arboviruses. Picaridin, oil of lemon eucalyptus (OLE), and IR-3535 are effective alternatives. OLE is not recommended for children younger than 3 years.

Repellents can also be applied to clothing, shoes, and camping gear, including bed nets, to increase effectiveness. However, permethrin is the most effective for this purpose and will reduce both mosquito and tick bites. It should not be applied to the body surface.

The US Environmental Protection Agency does not recommend any additional precautions for pregnant or lactating women or children using registered repellents (58). For children younger than 2 months, physical measures should be used to protect them from insect exposure. Specific recommendations for safe and effective use of repellents are summarized in Table 4.

No special infection control or isolation precautions are required for patients with alphavirus infections either in the community or in health care settings. Recently infected people may not be permitted to donate blood, tissue, or organs for transplantation.

EASTERN EQUINE ENCEPHALITIS VIRUS

Virology

Classification and Composition

EEEV was first isolated in 1933 after an equine epizootic in New Jersey and Virginia, though outbreaks had been occurring in horses for more than a century prior to that, and then identified as a cause of human encephalitis in 1938. Two antigenic varieties of North and South American origin had previously been recognized, but the South American strains have been re-designated as MADV, a new species in the EEEV complex (15). Genetically, the EEEV strains in

North America are relatively homogeneous and stable, although enzootic foci are segregated to a degree. Viral movement within the continent and selective pressures over a longer time frame have maintained the virus in one or two phylogenetic groups over the entire >50-year period during which strains have been available for study (Fig. 1).

Host Range

Severe, often lethal, CNS infection occurs in humans, horses, and pheasants. In contrast, whooping cranes, turkeys, and emus develop rapidly fatal viscerotropic infection, with necrotic lesions variously in spleen, liver, pancreas, intestines, kidneys, adrenals, and lungs, without CNS lesions. Other patterns of organ tropism have been described for various avian and mammalian species.

Devastating outbreaks have occurred in commercial pheasant, partridge, turkey, and emu flocks. Infection is rapidly spread among pheasants by pecking and probably by preening as well, because virus can be isolated from quills for as many as 6 days after experimental inoculation, and the birds can be infected orally. An outbreak in captive whooping cranes had nearly disastrous consequences, killing 7 of 39 of the endangered birds. The episode was an exception to the rule that native birds generally are resistant to infection, while exotic (introduced) species, such as sparrows, pheasants, and emus, develop lethal infections. The copious bloody diarrhea in ill whooping cranes and emus probably contributes to direct bird-to-bird transmission.

Horses have been considered dead-end hosts because viremia levels usually are too low to infect mosquitoes, though occasional horses may achieve circulating virus in the range of $10^{3.5}$ to $10^{5.5}$ PFU/ml, and experimental horse-to-horse transmission by *Aedes sollicitans* mosquitoes has been shown.

Epidemiology

(i) Distribution

The virus is transmitted only in the western hemisphere, where disease in horses or humans has been reported as far north as the Ontario and Quebec provinces in Canada, as far west as Wisconsin and eastern Texas, in the Caribbean, and in Mexico (59, 60). In the United States, transmission is concentrated in coastal areas on the Atlantic seaboard and Gulf Coast, but certain relatively constant inland foci of transmission also have been recognized (Fig. 5) (61).

(ii) Incidence and Prevalence of Human Infection

In the United States, infections are rare, sporadic, and steadily declining (7), with an average of 8 cases of neuroinvasive disease cases reported annually over the last decade (62). Subclinical infections typically have been found in only 0.05 to 0.17% of persons surveyed in epidemic areas, so levels of immunity in human populations remain low (63).

In Florida, equine and human cases are reported throughout the year, although nearly all human cases have had onset between June and September (Fig. 7) (64, 65). In mid-Atlantic and New England states, human cases usually occur no earlier than July and can appear through the end of October or until the intervention of cold weather (66). Heavy rainfall in the preceding year and heavy late summer precipitation have been associated with increased risk of EEEV infection (60). Increased rainfall, creating a high water table, augments the breeding habitat of *C. melanura*, and late summer rains expand the population of bridging vectors at an opportune stage for epizootic viral transmission.

(iii) Epidemic Patterns

Few outbreaks have been reported, and outbreaks are typically preceded by an epizootic in horses. An early Massachusetts outbreak led to 34 cases and, typical of EEE

FIGURE 5 Reported cases of EEE among humans in the United States, 2004 to 2013. The reported incidence is highest in Florida, where equine cases are reported perennially from the northeastern coast and throughout the peninsula. Relatively constant inland foci of transmission have been identified in upstate New York, southwestern Michigan, northeastern Indiana, and southcentral Georgia. In Massachusetts, human cases have been reported almost entirely from the eastern counties and, in New Jersey, from the southern counties. Reprinted from the CDC at http://www.cdc.gov/easternequineencephalitis/tech/epi/html.

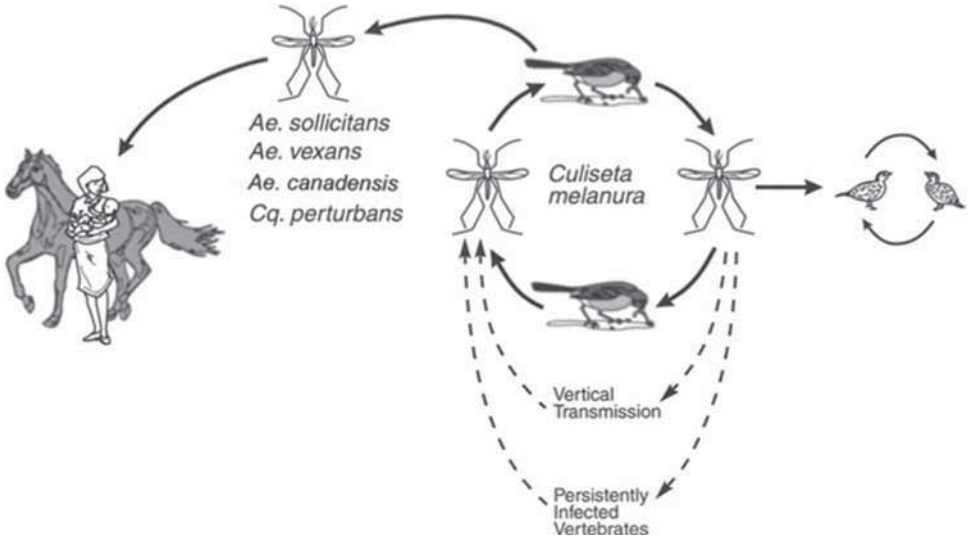

FIGURE 6 Schematic diagram of the EEE transmission cycle; solid lines show known portions, and broken lines show speculative portions. The principal enzootic mosquito vector, *Culiseta melanura*, transmits the virus among birds and occasionally initiates an outbreak among pheasants or other captive birds. Various other species bridge the enzootic cycle to infect humans and horses, which are dead-end hosts; the principal species include *Aedes sollicitans*, found in salt marsh coastal habitat; *Aedes vexans*, associated with open meadows and flooded ground pools; *Aedes canadensis*, associated with woodland pools; and *Coquillettidia perturbans*, found in open freshwater swamps with emerging vegetation. The viral overwintering mechanism is unknown but potentially includes vertically infected mosquitoes, persistently infected birds, and other vertebrates.

epidemics, was preceded by an epizootic of 300 equine cases in the state and simultaneous epizootics among horses in Rhode Island and Connecticut and an epornitic among pheasants the same summer (67). The last outbreak of note occurred in New Jersey in 1959, when 32 cases (22 fatal) were reported, yielding an epidemic attack rate of 101/100,000 (63).

Human infections are usually preceded by cases in horses. Immunization has lessened the predictive value of equine deaths, but pheasant, partridge, pig, and goat deaths also may signal a risk for human cases.

(iv) Transmission

The potential for viral transmission generally corresponds to the freshwater woodland swamp distribution of *Culiseta melanura* mosquitoes, the principal enzootic vector, with passerine birds as the major vertebrate hosts (60, 68). Its larval stages breed in depressions of mucky peat soils principally associated with upland red maples, or further inland, where Atlantic white cedar swamps drain into salt marshes, and in the South, with loblolly bay vegetation.

In the Caribbean, horse cases and enzootic viral transmission have been widely reported, and outbreaks of human disease have been reported from Jamaica and the Dominican Republic (69). The enzootic vectors appear to be *Culex taeniopus* in the Caribbean.

In North America, the virus is transmitted in a freshwater swamp-enzootic cycle among birds and strictly ornithophilic *C. melanura* mosquitoes (Fig. 6). Viral transmission is concentrated at the edge of, and within, the swamp interior. Other mosquito vectors that feed on both birds and mammals are required to bridge the enzootic cycle, carrying the virus to locations where humans, horses, and other susceptible dead-end hosts are exposed. Several species have been identified as bridge or epizootic vectors (Fig. 6). The virus also has been isolated from *A. albopictus*, an Asian mosquito introduced into the southeastern United States; however, its role in transmitting infections to horses or humans has not been shown.

Viral amplification through the summer initially is manifested by rising viral infection rates in *C. melanura*, followed by an increasing population of infected epizootic vectors (60). Infections in *C. melanura* with Highlands J virus (HJV), a benign WEEV-related virus transmitted in the same enzootic cycle, often foreshadows other indicators of EEEV transmission.

The permanence of EEEV foci has suggested a local overwintering reservoir, but attempts to demonstrate vertical transmission in *C. melanura* have not been convincing. Observations in enzootic coastal foci in New Jersey suggest that persistently infected permanent-resident birds might carry the virus through the winter, with the intervention of an unknown amplification mechanism preceding transmission in the *C. melanura* cycle. The maintenance of

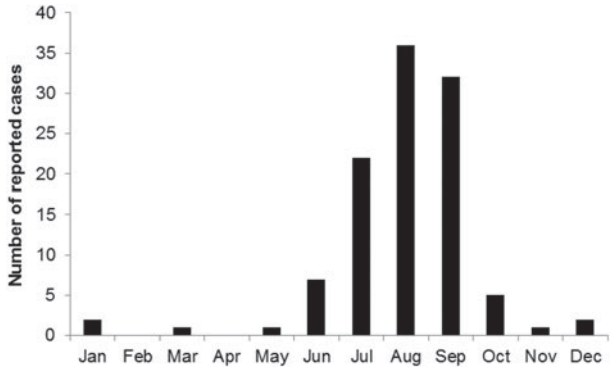

FIGURE 7 Reported cases of EEE by month, United States, 2003–2015. Data from reference 65.

temperate overwintering foci also is supported by genetic analyses of viral strains from disparate locations.

EEV is on the US *Select Agents and Toxins* list due to bioweapon potential, which does not include the South American subtypes now classified as MADV (70).

(v) Risk Factors

Young children and the elderly are most likely to develop illness (63, 66). In the 1959 New Jersey outbreak, infections occurred equally in all age groups, but clinical attack rates were higher in children < 4 years old and in adults > 55 years old. Respective inapparent-to-apparent case ratios were 8:1 and 16:1, whereas at intermediate ages the ratio was 29:1 (63). Factors underlying increased biological susceptibility at the extremes of age have not been defined.

Many patients have a history of residence near, or exposure to, tidal or freshwater swampy locations where epizootic vectors can be prevalent. However, the movements of birds and vectors from these habitats into adjoining suburban residential areas place people over a much wider area at potential risk. Horse ownership and occupational exposure to infected pheasants have not been associated with increased risk of acquiring illness or infection. However, emu owners and veterinarians may be infected when exposed to highly infectious bloody discharges or organs while nursing or handling ill or dead animals. Brains of horses that die of acute EEE also may contain high viral titers, placing veterinarians at potential risk. Four laboratory-acquired infections, two by aerosol exposure, have been reported (51).

Pathology

In fatal human cases the brain appears swollen, with flattened convolutions and narrowed sulci, while the meninges are congested (71–73). Inflammatory infiltrates of lymphocytes, histiocytes, and neutrophils are present in a perivascular distribution and in the parenchyma of the cerebral cortex, lentiform nuclei, thalamus, and brain stem, particularly in gray matter regions. The spinal cord may be affected at all levels, with lesions principally in the gray matter, but this is unusual. There is a mild to moderate loss of neurons, with neuronophagia, microglial reaction, and loss of oligodendrocytes with demyelinated lesions. Infection occurs preferentially in neurons, with occasional involvement of perivascular macrophages (72). Demyelinating lesions are more prevalent in patients dying after a prolonged illness.

Clinical Manifestations

The incubation period has not been defined but is likely to be in the range of 3 to 10 days. The initial descriptions of cases, principally in infants, emphasized the dramatic onset and rapid evolution of neurologic symptoms leading to coma and death in 30 to 75% of cases (73, 74). However, in many cases in children and adults, a prodromal illness of fever, chills, malaise, and myalgia occurs over 1 to 2 weeks, after which patients may recover or progress to more severe illness requiring hospitalization (69, 74, 75). Early symptoms may also include headache, photophobia, and dysesthesia. Those who deteriorate develop worsening headache, dizziness, vomiting, lethargy, and, later, neck stiffness, confusion, and convulsions. These events are compressed in infants, in whom illness begins with a rapid elevation of temperature, irritability, vomiting, and diarrhea, followed quickly by reducing conscious state or intermittent convulsions, leading to coma in 24 to 48 hours.

High fever, often above 39°C, is common, and the level of consciousness spans a spectrum from mildly depressed to comatose. Meningismus is common. The initial cranial nerve and motor examination may be normal, but, more frequently, abnormalities, such as nystagmus, gaze deviation, or sluggish pupils, are present, and alterations in tone, flaccid or spastic paralysis, and abnormal reflexes are present or evolve over several days. Some patients exhibit unilateral seizures, hemiparesis or hemiplegia, emotional lability, or aphasia reflecting focal damage. Clinical signs and radiological evidence of cerebral edema are frequent, and brain swelling and uncal or subtentorial herniation occurs. Hyponatremia is common and can be severe due to inappropriate antidiuretic hormone secretion.

Patients with a prodrome of more than 4 days preceding the onset of neurologic symptoms have a better outcome than those with a more rapid clinical course, suggesting that the early systemic immune response may modulate neuroinvasion and neurologic disease (69, 74–76). This is supported by one unusual case in which a patient with preexisting MAYV antibodies had only a systemic febrile illness without headache or neurologic signs (76), suggesting partial cross-protection. A mild case with limb dysesthesia and weakness, but no disturbances of consciousness suggesting encephalitis, indicated the possibility of myelitis without brain infection (76).

(i) Outcomes and Complications

Progressive neurologic deterioration leads to death in as many as 75% of encephalitis cases, usually within 10 days. Fatality rates are highest in the elderly, intermediate in children, and lowest in middle-aged adults (63, 66, 75). Although children are more likely to survive the illness than adults, infants are most likely to have residual neurologic abnormalities and to suffer permanent sequelae of motor weakness, paralysis, aphasia, intellectual disability, and continued seizures.

(ii) Laboratory Abnormalities

The peripheral leukocyte (WBC) count may be normal but more often ranges from 15×10^9 to 35×10^9/liter, with a neutrophil predominance and left shift (69, 74, 75, 77). The cerebrospinal fluid (CSF) may be xanthochromic and show increased pressure. The CSF WBC count is greatly elevated (10×10^6 to $2{,}000 \times 10^6$/liter), with a predominance of polymorphonuclear cells early in infection, followed by a mononuclear cell predominance. The ratio of mononuclear cells increases over several days, but in individual cases, a neutrophil pleocytosis has persisted into the second week of illness. Red blood cells are usually present, the protein content may be elevated, and the ratio of CSF to serum glucose is reduced to below 50% in half of cases. Magnetic resonance imaging is more sensitive than computed tomography scans; the majority of patients show thalamic, basal ganglia, and/or brainstem changes, with less frequent cortical meningeal and periventricular abnormalities, which generally improve over several weeks (77). Electroencephalogram (EEG) tracings show background slowing, with focal slowing in 50% of cases.

(iii) Differential Diagnosis

The diagnosis should be considered in the summer or early fall in persons with a history of exposure to an enzootic focus, particularly in the context of recent mosquito exposure. Public health surveillance data can shed light on current transmission levels (60). The principal considerations

in the differential diagnosis are bacterial cerebritis/cerebral abscess, herpes simplex encephalitis, or other viral encephalitides.

Laboratory Diagnosis

The virus has been isolated from serum 2 days after onset of illness in at least one case, but that is unusual (76). Virus often has been recovered from brain specimens of patients with fatal illness, and viral antigen has been demonstrated by immunohistochemical staining in brain biopsy and autopsy specimens (77). RT-PCR assays are more sensitive than cell culture and should increase diagnostic sensitivity (78) for blood, CSF, and premortem and postmortem tissues. Detection of EEEV IgM in serum is suggestive of recent infection, especially if accompanied by IgG seroconversion or a rise in titer. Detection of specific IgM in CSF is highly suggestive of encephalitis.

Prevention

Although, in most years, no more than one EEE case will occur in any single state, even the threat of a case can cause great concern in the general population. Public anxiety about EEE has led to reduced tourism in seaside resorts and to concern among owners of valuable racehorses. Thus, surveillance and preventive mosquito control programs are maintained in many areas with enzootic transmission. Personal protective measures are advisable when entering marshy and woodland areas where the enzootic cycle is maintained, bearing in mind that important epizootic vectors bite during the day as well as in the evening. Infants are less able to defend themselves against mosquitoes and should be kept under screened bassinets when outdoors.

An inactivated vaccine is available on an investigational basis for laboratory workers and others at high risk. Commercial, inactivated equine vaccine formulated with WEEV and VEEV offers a high degree of protection for horses, but annual boosters are required to maintain immunity.

Treatment

No specific antiviral therapy is currently available. Two case reports suggest that the use of intravenous immunoglobulin may have improved the patient outcome (79, 80). However, the reduction of the EEE mortality rate in the US, from 50% between 1955 and 1971 to 33% between 1972 and 1989, most likely reflects the lifesaving effects of high-level supportive care including respiratory support (66).

MADARIAGA VIRUS

MADV was original classified as EEEV but, as described above, has been redesignated a new species in the EEE complex (15). The virus has been shown to occur as three lineages in Central and South America and extends as far south as central Argentina (81). The virus has largely been associated with equine disease (82), although occasional human infections have been reported (81, 83, 84). Antibody prevalence rates have been highly variable, from 0 to 19%, depending on location (81). The lack of virulence of the Latin American subtypes in humans may be related to their greater sensitivity to human type I interferons (17).

Viral transmission in South America occurs in forests among *Culex (Melanoconion)* spp., especially *Culex taeniopus*, and rodents, marsupials, and, to a lesser degree, birds. Sporadic equine epizootics, often in conjunction with VEEV, have been reported, and the virus has been shown to cause severe encephalitis in South American camelids (85). A wide range of bridging vectors is probably involved in transmission to humans and other mammals. People appear to be regularly exposed but rarely seroconvert, presumably because the South American strains that circulate there are poorly infectious for humans (28).

WESTERN EQUINE ENCEPHALITIS VIRUS

Virology

WEEV was identified in 1930 as a cause of equine encephalitis in California and was isolated in 1938 from the brain of an encephalitic child. WEEV and SINV are the only members of the WEE antigenic complex known to cause human infections (Table 1). Several antigenic subtypes or varieties have been recognized, including an enzootic WEEV strain transmitted in subtropical Argentina (AG80-646), which differs in mouse pathogenicity and other biological characteristics (86). Genetic similarities of North and South American strains in the principal WEEV lineages indicate that the virus has moved between the continents.

Amino acid and nucleotide sequences of the viral capsid and envelope proteins are most similar to those of EEEV and SINV, respectively, indicating that WEEV is a recombinant of EEEV and an ancestral New World Sindbis-like virus (12). The recombinant event is speculated to have occurred thousands of years ago, before the divergence of EEEV strains into North and South American varieties, and presumably occurred within the avian-mosquito transmission cycle shared by all three viruses.

Host Range

Humans, horses, and emus develop clinical symptoms after natural infection. In the eastern United States, HJV, transmitted in the same enzootic cycle as EEEV, is a rare cause of equine encephalitis but has produced outbreaks of fatal illness among pheasants, turkeys, and chukar partridges. Four human HJV infections based on IgM detection have been reported, though all were also infected with St. Louis encephalitis virus, which was the likely cause of their encephalitis (87). Captive exotic emus die of a fulminant viscerotropic infection, similar to that caused by EEEV. Experimental infection produces antibody without illness or viremia in cattle, illness without encephalitis in burros, and neurologic infections in a small proportion of ponies. Fatal illnesses are produced in certain wild rodents. The Argentine enzootic strain is avirulent in horses and exhibits minimal neurovirulence in mice (86).

Epidemiology

(i) Distribution

WEEV activity has been reported from the western United States and Canadian provinces, Mexico, Guyana, Brazil, Argentina, and Uruguay (88–90). Small equine outbreaks without human disease have occurred in Brazil and Guyana. In the temperate provinces of Argentina, intermittent epizootics have occurred in an area between latitudes 28° and 40°S, and from the Andes to the Atlantic coast, spilling over into adjacent areas of Uruguay. The enzootic WEEV subtype is transmitted in the subtropical Chaco province (86).

(ii) Incidence and Prevalence of Human Infections

Subclinical infections are common among residents of rural areas where the viral transmission cycle is maintained. Regular sporadic and occasional epidemics of human disease

occurred in the US up to the mid-1980s, but human cases have become rare in recent decades. The US Geological Service did not report any human cases between 2003 and 2015 (65). Estimates of the ratios of apparent to inapparent cases have ranged from 1:1 for children < 1 year old to 1:58 for children 1 to 4 years old to 1:1,150 for adults (7). In an outbreak where seroconversions were monitored, the overall ratio of reported cases to infections was 1:160.

(iii) Epidemic Patterns

WEEV now occurs as a very rare sporadic infection in the western United States and Canada, with only five cases reported since 1988 and none since 2003. The virus is also now rarely detected in mosquitoes or sentinel animals or mosquitoes, the last found in 2007 for the former and in 2013 for the latter (65). However, past outbreaks have resulted in thousands of cases, and the epidemic potential of the virus should not be overlooked. In the largest recorded outbreak, in 1941, 3,400 human and hundreds of thousands of equine cases were reported from Minnesota, North and South Dakota, Nebraska, Montana, Alberta, Manitoba, and Saskatchewan, with an estimated incidence of 167/100,000 in North Dakota (89). Between 1945 and 1958, more than 600 cases were reported from California, 375 in 1952 alone, with an incidence rate of 340/100,000 in rural Kern County, and, in 1975, a regional outbreak produced 145 cases in Manitoba and 132 cases in the Red River Valley of North Dakota, with an estimated attack rate of 11/100,000 residents (90, 91). The last major outbreak, in 1987, led to 41 cases in the Great Plains and mountain states and 30 cases in Colorado. Active case finding in the state found an incidence of 1.6/100,000 in affected counties (88). Incidence rates consistently are highest in rural areas where the viral transmission cycle is maintained, with a lower risk in town and urban residents.

Previously, seroprevalence rates of 20% were common in areas of the western United States. However, a decline in the rural population and changes in land-use patterns and lifestyles have reduced the risk of acquiring infection, such that current antibody levels in some areas of endemicity are in the range of 1%. Few human cases have been reported from South America, despite active surveillance in areas experiencing epizootics in horses. The epizootic strains appear to be virulent variants of enzootic strains (86). Clusters of human cases were reported in 1972–1973 and again in 1982–1983; a total of seven cases, with one death, were recognized, for an estimated attack rate of 3/1,000 inhabitants.

(iv) Transmission

In the western United States, the virus is transmitted between *Culex tarsalis* mosquitoes and passerine birds, principally sparrows and house finches. *C. tarsalis* is adapted to naturally flooded ground pools and irrigated pastures, found mainly in rural and agricultural areas where most human and equine cases are acquired. Viral amplification and epidemic risk are increased by heavy winter snowpack, spring precipitation, and flooding, which expand vector larval habitat. Another cycle involving *Aedes melanimon* and the blacktail jackrabbit has also been identified (7). Concurrent outbreaks of WEEV and SLEV, transmitted in the same enzootic cycle in the western United States, have been reported frequently. However, extrinsic incubation of SLEV in vector mosquitoes is slower and requires higher temperatures, delaying transmission, often after WEEV activity has subsided.

(v) Risk Factors

A bimodal age distribution of risk has been observed, with a sharp increase in risk with advanced age and a secondary peak in infants (88). Residence in rural areas is a principal risk factor; however, the long flight range of the mosquito vector allows it to infiltrate towns and suburban areas, and, often, more cases are reported from these populated locations, while population incidence rates are higher in thinly populated rural areas (91). In semiarid locations, WEEV incidence is highest in irrigated areas along the major river basins. Incidence rates in the United States and Argentina have been higher in males, probably reflecting greater outdoor exposure in ranch and farming activities. Cases occasionally are reported among persons engaged in outdoor leisure activities. Seven laboratory-acquired cases, including two deaths, have been reported through 1980, among them a well-documented case resulting from conjunctival or respiratory infection (92, 93).

Pathology

The brain appears normal or exhibits a moderate degree of vascular congestion (30, 90, 94–96). There is a mild patchy, sometimes extensive, infiltration of the meninges and prominent vascular congestion. Inflammatory infiltrates of lymphocytes, plasma cells, and neutrophils are found in a perivascular distribution, with invasion of the vessel wall and vascular necrosis in some instances. Parenchymal lesions are typified by widely scattered, discrete foci of tissue necrosis with microglial proliferation and inflammatory cell infiltration. Neurons are found in various stages of degeneration. Lesions are distributed mainly in the subcortical white matter, the internal capsule, thalami, basal ganglia, substantia nigra, dentate nucleus, and molecular layer of the cerebellum and gray matter of the brain stem and spinal cord. Widely scattered focal areas of demyelination are seen. Infections acquired in infancy have led to significant disturbances in brain development resulting in cerebral atrophy and demyelination, with the formation of multiple glia-lined cysts and vascular calcification (94, 95).

Clinical Manifestations

The reported incubation period has been as short as 2 days in one case and, in two laboratory-acquired infections, it was 4 days in one and up to 10 days in the other (92, 93). Illness typically begins with the sudden onset of headache, usually followed by dizziness, chills, fever, myalgia, and malaise (89, 90, 92, 93). These progressively worsen over a few days and may be followed by dizziness, tremor, irritability, photophobia, and neck stiffness. Patients typically appear to be drowsy or may be restless, with a moderately elevated temperature. Meningismus is present in about 50% of cases. Neurologic abnormalities are usually limited to weakness and generalized tremulousness, especially of the hands, lips, and tongue. Cranial nerve palsy, motor weakness, spasticity, or convulsions occur in < 5% of patients. Hemiplegia, quadriplegia, and focal weakness or seizures are unusual (∽ 3% of cases). Depressed conscious state or coma develops in < 10% of cases, with respiratory failure in some cases. Hyponatremia occurs occasionally but less frequently than with EEE. General improvement begins several days after the resolution of fever, typically within a week to 10 days after the onset. Cases of mild illness have been described in which fever, headache, and afternoon fatigue persist for several days to a week, without more serious manifestations.

In infants, the initial presentation and clinical progression are more rapid, evolving from a nonspecific illness of fever, irritability, and diarrhea to convulsions and coma (94, 95, 97, 98). Seizures usually are generalized but may have a focal component. Increased muscular tone, often to a point of generalized rigidity, is typical. Rapid fatal outcome or significant residual brain damage was common among cases reported from the 1940s to 1950s.

(i) Outcomes and Complications

The overall mortality rate is 4% and is highest among persons >75 years old. Signs of parkinsonism were reported on follow-up in at least 11 cases, though there is no evidence that WEEV infection is a significant cause of Parkinson's disease in endemic areas (93, 96, 99).

However, serious sequelae are more prevalent in recovered infants and children, with the most serious outcomes in the youngest infants (100). Among infants <3 months old at the onset of illness, 44% have had extensive brain damage resulting in serious neurologic sequelae, and >25% have had mental retardation necessitating institutionalization. Other sequelae included lesser degrees of developmental delay, convulsions, spasticity, and extrapyramidal movement disorders. Sequelae have been less severe and more likely to improve in older infants and children. Five cases of perinatal illness following late-trimester or postpartum maternal infection have been reported in which vertical transmission of infection was surmised. The neonates became ill 3 to 6 days after delivery, while maternal illness began on the same postpartum day in two cases and in the others had occurred 3 or 10 days prepartum (100, 101).

As nearly all of the clinical data are from case series occurring more than five decades ago, outcomes in modern health care settings should be better, as was seen with EEEV.

(ii) Laboratory Abnormalities

The peripheral WBC count is usually normal or elevated to 15×10^9 to 25×10^9/liter (89, 90). The CSF rarely is xanthochromic; the WBC count is usually elevated to 110×10^6 to $1{,}500 \times 10^6$/liter, with an early neutrophil and later mononuclear cell predominance. CSF protein is moderately elevated in about 50% of cases; glucose is usually normal. When recorded, CSF pressure was elevated in about two-thirds of cases.

Brain scans, cerebral angiography, and contrast-enhanced computed tomography findings have been unremarkable in the few cases in which they have been reported (29, 90, 102–104). One neonatal case showed diffuse low-attenuation lesions in the white matter, accompanied by multifocal EEG epileptiform discharges. Follow-up studies 4 months later showed severe diffuse seizure activity and enlarged ventricles, encephalomalacia, and numerous intracranial calcifications in the insular cortex and thalamus bilaterally. In several cases mimicking the focal presentation of herpes encephalitis, EEGs showed diffuse slowing with focal delta activity in the temporal region (29, 102–104).

(iii) Differential Diagnosis

Due to its overlapping geographic, seasonal, and transmission patterns, as well as its similar clinical features, SLEV infection is the principal alternative diagnosis (105). Even with the epidemiological clues of occurrence during a recognized outbreak, clinical differentiation of WEEV from other causes of acute CNS infection is difficult. Other clinical diagnoses that were later proved to be due to WEEV or SLEV are shown in Table 3.

TABLE 3 Initial diagnosis of diseases ultimately proven by laboratory test to be either WEE or St. Louis encephalitis[a]

Infectious causes
Polioencephalomyelitis
Bacterial meningitis
Brain abscess
Tuberculous meningitis
Mumps encephalitis
Coccidioidal infection
Lymphocytic choriomeningitis
CNS syphilis
Coxsackie encephalitis
Pneumonia
Otitis media
Subacute bacterial endocarditis
Sydenham's chorea
Noninfectious causes
Brain trauma
Cerebrovascular accident
Intracranial hemorrhage
Intracranial neoplasm
Convulsive disorder
Cardiac failure

[a]Data from reference 105.

Laboratory Diagnosis

Viremia is generally considered to have cleared before the onset of CNS signs, and virus is rarely recovered from blood. Virus was isolated from the CSF of only 2 of 21 patients in one series and, in two cases, from brain biopsy samples that were obtained to rule out herpes encephalitis (99). RT-PCR-based tests appear to be more sensitive than culture (78). Detection of virus-specific IgM in CSF or serum provides the most rapid laboratory confirmation. Serologic cross-reactions with other alphaviruses have not been a major concern in the western United States and Canada.

Prevention

Where WEE has occurred in epidemics and epizootics, public health programs have been established to survey viral activity in mosquitoes and birds and to preemptively control vector mosquitoes. An experimental inactivated vaccine is available on an investigational basis for laboratory workers and other persons at high risk of exposure. Horses in enzootic areas should be immunized on an annual basis.

Treatment

No specific therapy is currently available, and treatment is supportive, including respiratory assistance and anticonvulsant therapy. Favipiravir (T-705) and indole-2-carboxamide compounds have reduced disease severity in mice (106, 107).

VENEZUELAN EQUINE ENCEPHALITIS VIRUS

Virology

VEEV was first detected in an outbreak of equine encephalitis in Colombia and Venezuela in 1936 and only later was

TABLE 4 Precautions to minimize exposure to mosquitoes and ticks[a]

Use an insect repellent on exposed skin to repel mosquitoes, ticks, fleas, and other arthropods. EPA-registered repellents include products containing DEET at concentrations of 30 to 50%, which are effective for several hours.
For mosquitoes only: Preparations containing picaridin (7 and 15% concentrations), para-menthane-3,8-diol (PMD), oil of lemon eucalyptus (which contains PMD), or IR-3535.
DEET formulations as high as 50% are recommended for adults and up to 30% for children older than 2 months. Protect infants younger than 2 months by using a carrier draped with mosquito netting with an elastic edge for a tight fit.
When using sunscreen, apply sunscreen first and then repellent. Repellent should be washed off at the end of the day before going to bed.
Wear long-sleeved shirts (which should be tucked in), long pants, and hats to cover exposed skin. When you visit areas with ticks and fleas, wear boots, not sandals, and tuck pants into socks.
Inspect your body and clothing for ticks during outdoor activity and at the end of the day. Wear light-colored or white clothing so ticks can more easily be seen. Removing ticks right away can prevent some infections.
Apply permethrin-containing or other insect repellents to clothing, shoes, tents, mosquito nets, and other gear for greater protection. Permethrin should not be applied directly to the skin. Repellent is generally effective for several washings.
Stay in air-conditioned or well-screened housing, and/or sleep under an insecticide-treated bed net. Bed nets should be tucked under mattresses and can be sprayed with a repellent if not already treated with an insecticide.

[a]Based on reference 164 and Centers for Disease Control and Prevention recommendations (http://wwwn.cdc.gov/travel/contentMosquitoTick.aspx).

found in human encephalitis cases occurring during a period of epizootic activity. Several subtypes of VEEV comprise a complex of six antigenically related but epidemiologically and ecologically disparate viruses. Only type I (VEEV) and type II (Everglades virus [EVEV]) are known to cause human disease. VEEV is further subdivided into IAB, IC, ID, IE, and IF strains. Epizootic viruses (IAB, IC, and IE) emerge at unpredictable intervals of years or decades, producing extensive outbreaks in equines and humans. By contrast, the sylvatic VEEV subtypes (ID, IE, and IF) are transmitted continuously in forest or swamp foci, causing sporadic human infections, but in general no equine disease. Subtypes can be distinguished by special serologic procedures and by monoclonal antibodies; in addition, they differ biologically in characteristics such as plaque size, viremia levels, rapidity of clearance from blood, and host range (108, 109). In one Venezuelan focus, the close genetic relationships of sylvatic ID and epizootic IC viruses (<1% nucleotide substitutions in their entire genomes) suggested that the epizootic strain may have arisen by mutation from continuously circulating sylvatic viruses, and recent epizootic strains of IE have arisen from enzootic strains (18, 110). In contrast to spontaneous epizootics due to IC viruses, certain IAB virus outbreaks are speculated to have arisen from improperly inactivated equine vaccine, as the IAB strains isolated during many later epizootics were virtually identical to the Trinidad donkey and other early strains of IAB virus used to produce inactivated equine vaccine (19).

After the divergence of VEEV and EEEV, the VEEV lineage diverged into several enzootic lineages that generally occupy non-overlapping distributions in the tropical and subtropical areas. Of these enzootic lineages, one genotype of subtype ID virus evidently generated the epizootic strains implicated in outbreaks dating to the 1920s (subtypes IAB and IC) (Fig. 1) (111, 112). Geographically isolated sylvatic viruses such as subtypes II (EVEV-Florida) and IIIB (Bijou Bridge-Colorado) may have become established through introductions by migratory birds, with their subsequent adaptations to local enzootic cycles. The latter virus, presumably an introduction of Tonate virus from South America, is believed to have been a contemporary event, occurring only approximately 40 years ago.

Host Range

Illnesses and encephalitis in horses and burros have been fatal in 20 to 40% of animals, without apparent age-related susceptibility (108, 113). Goat deaths have been reported in outbreaks, and dogs occasionally die with evidence of CNS pathology. Experimental infections have produced high mortality rates in species of North American rodents, dogs, and coyotes. Beef cattle, pigs, some bats, rodents, and birds (especially herons) develop sufficient viremia to participate in viral amplification.

Horses experimentally infected with sylvatic viral strains develop fever, mild leukopenia, and an insignificant viremia, while epizootic strains produce an illness with high fever, severe leukopenia, depressed hematocrit, high viremia, and encephalitis (108). Equine virulence patterns generally are mirrored in experimentally infected English shorthaired guinea pigs, except for some enzootic subtype ID strains (18). Rhesus monkeys experimentally infected with epizootic strains develop febrile illness with elevated hepatic transaminases, while enzootic strains produce no symptoms or fever (114).

Epidemiology

(i) Distribution

Sylvatic viruses are transmitted in tropical and subtropical swamps and forested foci in North, Central, and South America. Epizootics have occurred principally in northern South America in Colombia, Venezuela, Trinidad, Peru, and Ecuador, but a series of outbreaks between 1969 and 1972 extended from Guatemala through all the countries of Central America, except Panama, and into Mexico and Texas (Fig. 8) (115).

(ii) Incidence and Prevalence of Human Infections

A low ratio of apparent to inapparent infections, 1:10, is typical; however, airborne transmission has resulted in illness in >75% of laboratory-infected persons (116). In the 1962 Zulia state outbreak, attack rates were 183.5/1,000/month in children <15 years old and 72.9 in persons >15 years old, reflecting immunity acquired during a series of outbreaks from 1942 to 1949. Six years later, in 1968, VEE recurred, and attack rates were highest in children <6 years of age. Infants <1 year old may be protected by maternal immunity. In the immunologically naïve Texas population, age-specific immunity rates after the 1971 outbreak were highest (21/100,000) in the 20- to 39-year age group, presumably reflecting outdoor exposure in occupational and leisure activities (117).

Outbreaks occur during the rainy season, the monthly distribution of cases varying with local and secular changes

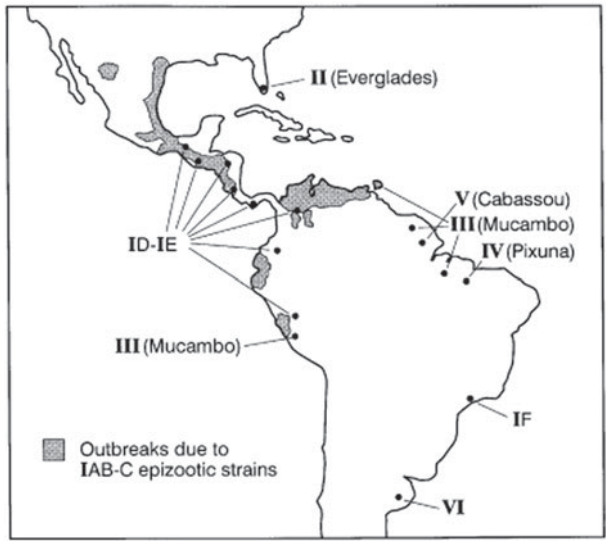

FIGURE 8 Geographic distribution of VEE epizootics and sylvatic viral subtypes.

in rainfall patterns. Outbreaks in northwestern Venezuela often have started in April, peaked in June or July, and subsided by December. Sylvatic viral strains are transmitted continuously in their tropical and subtropical foci.

(iii) Epidemic Patterns

Sylvatic viruses are transmitted among small mammals and aquatic birds in swampy and forested locations principally by *Culex melanoconion* mosquitoes (Fig. 9A). Sylvatic VEEV infections occur among persons living near, or entering, enzootic foci, e.g., among soldiers on jungle bivouacs (118–121). In certain locations, endemic transmission to local residents leads to age-dependent seroprevalence rates of >50%.

Epidemic VEE occurs in conjunction with epizootic transmission among horses, leading to combined outbreaks of tens of thousands of human cases and hundreds of thousands of equine cases (Fig. 9B) (112, 115, 117, 122). In the 1995 outbreak in Venezuela and Colombia, an estimated 85,000 human cases were identified, 3,000 with neurologic symptoms (4%) and 300 fatalities (110, 112). A serosurvey found that 36% of the resident population had been infected and as many as 8% of the state's equines had died. Similar statistics have been reported from previous outbreaks.

(iv) Transmission

A susceptible equine population is essential for epizootic emergence and spread. Horses are potent amplifying hosts, their high level of viremia facilitating rapid animal-to-animal transmission by a variety of mosquito vectors, including floodwater *Aedes taeniorhychus*, *Psorophora confinnis*, *Mansonia* spp., and *Deinocerites* spp. Other biting insects may transmit the virus mechanically. Outbreaks frequently occur in normally dry locations when heavy rainfall and flooding have expanded mosquito breeding habitats and mosquito and rodent populations. In areas with susceptible horses, outbreaks spread at rates of several kilometers per day, followed by associated human cases several weeks later (112, 115). Transmission continues inexorably until susceptible horses have been depleted by natural infection or immunization or until the onset of dry weather and diminishing mosquito numbers. Transmission sometimes has resumed with the return of the rainy season. The intensity of transmission and high attack rates among horses and humans create a high level of immunity that may contribute to the periodicity of outbreaks every 10 to 20 years

In outbreaks where few equine deaths were noted, the possibility of supplemental viral amplification in humans was considered. The viremia in humans is sufficiently high to infect mosquitoes, and peridomestic *A. aegypti* mosquitoes are potential vectors. In addition, direct person-to-person spread seems possible because virus can be isolated from the pharynx, sometimes on successive days, in 6 to 40% of acutely ill patients (123). However, no clinical or serologic evidence of household clustering or intrafamilial spread has been recognized, suggesting a minor role, if any, for person-to-person spread or for the importance of humans as amplifying hosts (112, 123).

Numerous laboratory outbreaks have been traced to airborne infection, with a total of 150 cases and one death reported through 1980 (116, 121). Very brief exposures in persons not working directly with the virus (e.g., walking through a laboratory) have been sufficient for infection to occur. The IAB and IC subtypes are on the US *Select Agents and Toxins* list due to bioweapon potential (70).

(v) Risk Factors

Outbreaks occur principally in rural areas where horses and burros still are commonly used as transportation. Native Indians have been at greater risk because of residence in rural rancherias and other factors related to increased mosquito exposure or horse ownership (112).

Pathology

Histopathological lesions have consistently been found in the CNS, lungs, heart, spleen, lymph nodes, liver, and gastrointestinal tract and, less often, in the kidneys and adrenal glands (37). The respiratory and intestinal mucosa appears hyperemic, with microscopic evidence of vascular congestion, hemorrhage, and vascular injury. Inflammatory reactions are most prominent in the lungs, with diffuse or patchy alveolar septal infiltrates, intra-alveolar edema, and, rarely, hemorrhage. Myocarditis and centrilobular hepatic necrosis may be seen. The brain appears swollen and hyperemic. Diffuse cellular infiltrates involve the meninges and, in one-third of cases, the brain and spinal cord. Encephalomyelitis is characterized by perivascular inflammatory infiltrates, hemorrhages, foci of neuronal degeneration, and glial proliferation. Lesions are distributed in the cerebral gray and white matter, especially in the substantia nigra. The lymph nodes and spleen exhibit extensive follicular necrosis, germinal center lymphoid depletion, neutrophil infiltration, lymphophagocytosis, and vasculitis.

Spontaneous abortions after VEE-like infections have been associated with massive cerebral necrosis and typical acute lesions in the aborted fetuses, including cerebral calcifications similar to those associated with toxoplasmosis in one case (124). Congenital VEEV infection has been confirmed virologically by recovery of the virus from aborted fetuses (74). Infections acquired early in pregnancy have been associated with fetal hydranencephaly, porencephaly, and cerebral dysgenesis, a pattern of lesions reproduced in experimentally infected fetal rhesus monkeys.

Clinical Manifestations

The incubation period has been characterized from laboratory and other outbreaks usually as 2 to 3 days, but ranging

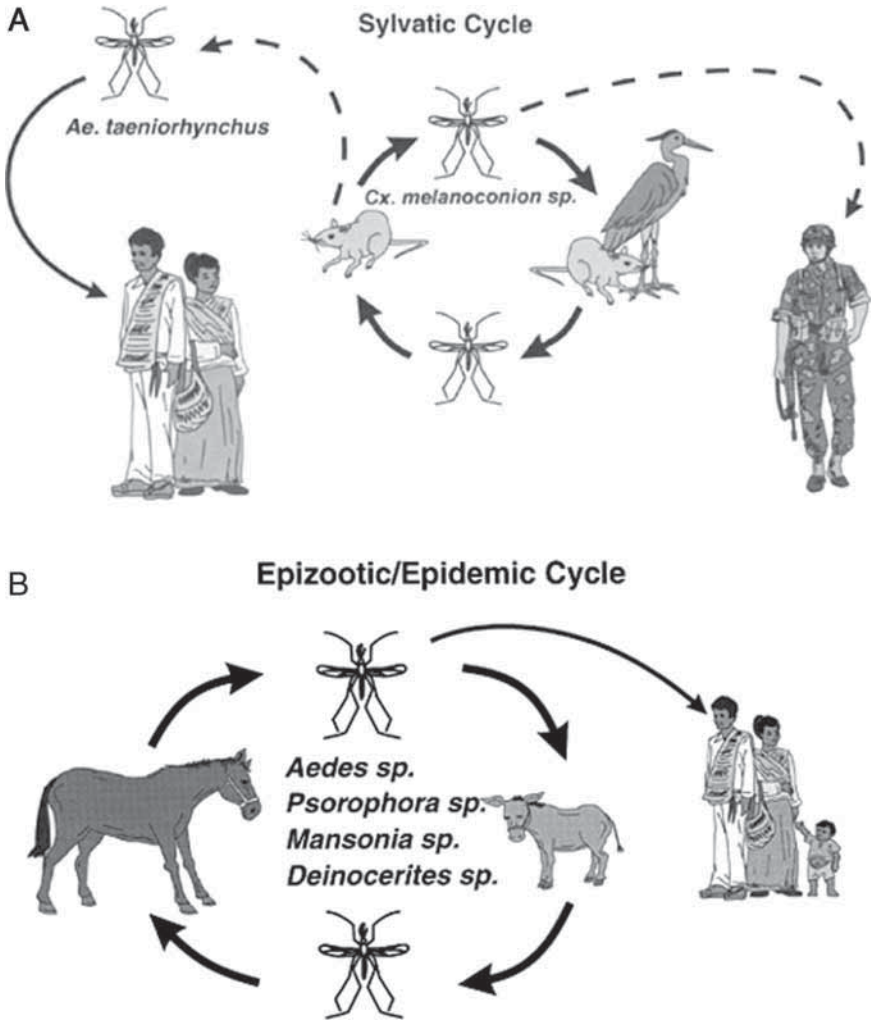

FIGURE 9 Life cycles of Venezuelan equine encephalitis virus. (A) Sylvatic VEEVs circulate continuously in silent tropical and subtropical foci among (*Culex Melaconion*) mosquitoes and small mammals or aquatic birds. Humans (e.g., soldiers on jungle bivouacs) are infected when they chance upon transmission foci. Bridging vectors (e.g., *Aedes taeniorhychus*) that feed on viremic vertebrates in a sylvatic focus can carry the virus to nearby areas of human activity. (B) In contrast to the continuous cycling of sylvatic VEEV subtypes, epizootic VEEV had never been isolated until recently, except during periodic outbreaks. Once introduced, epizootic viruses are rapidly amplified among equines (horses and burros). Equines develop high viremia levels, so various mosquito species can function as biological vectors (only some important species are shown), and other biting insects, such as blackflies, can spread the virus mechanically. Humans develop illness with high viremia levels but probably have an insignificant role, if any, in viral amplification. Transmission declines as susceptible equines are exhausted by natural infection or immunization. It is speculated that epizootic IC viruses arise by mutation from sylvatic ID viruses; the subpopulation circulates in a sylvatic cycle and under appropriate conditions is amplified, leading to epizootic and epidemic spread.

from <1 to 5 days (116). Well-documented cases have had onset 12 hours after laboratory exposure.

The onset of illness is abrupt and rapidly incapacitating, with sudden chills, headache, fever, body aches, and prostration (116–118, 120). Headache is nearly unbearable and may be exacerbated by even the smallest movements of the eyes or neck and is worsened by bright light. Asthenia, dizziness, and acute discomfort are incapacitating. Nausea and vomiting are common. The temperature is moderately elevated, and the face appears flushed, with injected or suffused conjunctivae. Pharyngeal pain and inflammation are accompanied by cervical and other lymphadenitis. There may be mild subcostal abdominal tenderness. Symptoms resolve over several days with defervescence, but a dull headache and weakness may persist several days longer. Occasionally, fever and symptoms recrudesce after an initial remission.

Despite the disease name, severe neurologic symptoms and encephalitis occur in only 4 to 14% of cases overall, principally in children and the elderly; however, tremulousness and somnolence, suggesting milder neurologic involvement, are not uncommon in a larger proportion of cases (74, 112, 115, 122). The risk for neurologic infection in children, compared to adults, has been estimated to be 10-fold greater (4% of all symptomatic cases versus 0.4%). Among patients hospitalized with neurologic symptoms, as many as 85% are children <10 years old, 15% are adults >50 years old, and only 1% are young adults (112). Neurologic symptoms often appear late in the illness, sometimes after defervescence. Seizures are common and

have a focal component in 40% of cases (112, 125). Cranial nerve palsy, motor weakness, paralysis, and specific signs of cerebellitis are present in 5 to 10% of hospitalized patients, while stupor and coma occur less frequently. More than half of hospitalized patients may exhibit clinical signs of elevated intracranial pressure. Meningismus is found in the majority of childhood cases (75, 112, 115).

(i) Outcomes and Complications

The fatality rate among encephalitis cases is 10 to 25%, with higher mortality rates in children <4 years old. Overall, between 0.2 and 1% of all symptomatic cases are fatal, nearly all in children. The outcome of recovered cases has not been well characterized; however, forgetfulness, nervousness, asthenia, and headache are not uncommon, at least through the first year. Recurrent seizures, motor impairment, psychomotor retardation, and behavioral disorders are sequelae in children (126).

Sylvatic EEEV infections generally cause a mild and self-limiting febrile illness, though leukopenia is common and occasionally severe (118). Fatal encephalitis occurred after Tonate virus (IIIB subtype) infection in one study (127). All three recognized cases of subtype II (EVEV) infection produced encephalitis and coma without sequelae.

(ii) Laboratory Abnormalities

Leukopenia is characteristic, resulting from an early lymphopenia that rebounds over a week, while the absolute neutrophil count moves in the opposite direction, declining to 0.5×10^9 to 2×10^9/liter after 5 to 7 days. The net result is a declining total WBC count in the range of 2×10^9 to 6×10^9/liter (117). CSF contains a variable pleocytosis, usually with a lymphocytic predominance. Elevated CSF glucose has been reported; CSF protein typically is normal and rarely is elevated. About one-third of patients have had moderately elevated serum liver transaminases. Volunteers receiving live attenuated vaccine have exhibited transient attenuated or depressed T waves on electrocardiograms.

(iii) Differential Diagnosis

Sylvatic infections are indistinguishable from many ordinary or tropical viral infections, such as Oropouche virus and other bunyavirus fevers, while the absence of a rash and joint symptoms helps to rule out MAYV disease. The neurologic syndrome cannot be differentiated from that due to EEEV, SLEV, or other pathogens that occur in the same locations. Even in the context of an epidemic, the clinical diagnosis based on symptoms of acute febrile illness has been accurate only in about two-thirds of cases. The absence of rash and hemorrhagic phenomena aids in excluding epidemic dengue. An ongoing equine epizootic is the most helpful clue to the diagnosis.

Laboratory Diagnosis

Virus can be recovered from blood most easily in the first 3 days of illness, with declining sensitivity up to the eighth day (121, 123). Viral titers remain in the range of $10^{3.8}$ to $10^{5.7}$ PFU/ml until at least the fourth day of illness. In addition, virus can be recovered from the pharynx in 7 to 40% of cases in the same time frame (122, 123). RT-PCR appears to be highly sensitive in detecting virus in acute-phase serum samples (128). Viral isolations also have been made from decidua of abortions and from bone marrow but have been inconsistent from brain (5). The subtype of viral isolates should be rapidly identified to inform public health decisions on control measures.

In-house serological tests are available in reference laboratories. Detection of virus-specific IgM in acute-phase serum provides a rapid presumptive diagnosis in both horses and humans (101, 129). No cross-reactions with WEEV or EEEV antibodies are detected with available IgM and IgG enzyme-linked immunosorbent assay kits using IAB viral antigen. Antibodies to epizootic viruses can be differentiated from antibodies to enzootic viruses by using an epitope-blocking enzyme immunoassay (EIA) (130). Neutralization (NT) titers may also provide subtype specificity of the antibodies. Specific IgM has persisted for several months after immunization with TC-83 vaccine, but its longevity after natural infection is unknown.

Prevention

Personal protection is based on avoiding foci of enzootic and epidemic transmission, using mosquito repellents, and wearing protective clothing.

Systematic equine immunization should dampen epizootic transmission and, in principle, can prevent the emergence of outbreaks. Only the live attenuated TC-83 vaccine should be used, to avoid risk of inadequate inactivation and iatrogenic transmission. Attenuated equine vaccine is no longer produced in the United States, but production and distribution are maintained in support of public veterinary vaccination programs in several Latin American countries.

Live-attenuated TC-83 strain and killed (TC-84) experimental human vaccines have been used extensively to protect laboratory workers and others at high risk of exposure (131). Direct studies of the former's efficacy are lacking, but effectiveness with its use can be inferred from the reduction of laboratory-acquired cases and from experimental-challenge experiments in which vaccinated animals have been protected against airborne challenge infection. A single dose produces a neutralizing antibody response in 82% of vaccinees and induction of specific IgA, with 56% retaining a neutralizing antibody titer of >1:20 for 10 years (132). The TC-83 strain is only partially attenuated, and self-limited influenza-like illness similar to those of natural infection occurs in 20% of vaccinees (133). Virus has been recovered from blood and pharyngeal swabs as in the natural infection; therefore, immunosuppressive conditions or pregnancy in either the intended vaccinee or household contacts is a reason for exclusion.

Among the 18% who fail to respond serologically, 76% seroconvert after reimmunization with the killed TC-84 vaccine (132). Preexisting antibodies from prior alphavirus vaccinations may interfere with a proper immune response, and other vaccinees show a persistent failure to respond to the live vaccine (134). Although vaccination with inactivated TC-84 vaccine is recommended in live-vaccine nonresponders, inactivated vaccine may not protect against infection by the respiratory route, the principal concern in laboratories. In addition, titers of TC-84-induced antibody to enzootic ID and IE subtypes are lower, and these infections have occurred in vaccinees. Because of the limitations of available vaccine, all laboratory manipulations should be undertaken in biosafety level 3 (BSL-3) facilities. Other vaccines are under development.

Nonreplicating VEEV replicon particles have shown promise as a vehicle for delivery of chimeric vaccines for a range of other infectious agents.

Treatment

Treatment is symptomatic, with analgesics and bed rest in mild cases and supportive therapy in more severe cases. Fatality rates in children with neurologic symptoms remain high, in part because outbreaks occur in relatively undeveloped rural areas. Pneumonia is the principal nonneurologic illness, with evidence of secondary infection in some cases. In addition, significant lymphoid depletion could predispose to bacterial infection through the gastrointestinal tract. Appropriate early antibacterial therapy is important in acutely ill patients.

CHIKUNGUNYA VIRUS

Virology

The name of CHIKV derives from a Kimakonde root verb, *kungunyala*, meaning "to dry up or become contorted," specifically modified in early times to describe the bent posture of patients with painful joints (135). The disease has occurred for at least decades over a wide geographic area from sub-Saharan Africa, where genetic studies suggest the virus originated, to the Philippine archipelago in Asia, where it was transported during the late 19th or early 20th century (136). The West African strains form one major enzootic genetic lineage, while the other major enzootic lineage (East, Central, South African [ECSA]) has spread widely to generate the Asian (AL) and Indian Ocean (IOL) epidemic lineages, with some phenotypic and antigenic differences (137). Individual epidemics may be caused by specific genetic variants (138). The different CHIKV lineages and sublineages do not have clear pathogenic differences.

Host Range

Disease has not been observed in naturally infected animals. Monkeys and baboons, the principal vertebrate hosts in the African enzootic viral transmission cycle, develop a viremia after infection but remain asymptomatic. Viremia of a sufficient level to potentially support viral transmission has been experimentally produced in some species of rodents and bats.

Epidemiology

(i) Distribution

Viral transmission has been reported within a vast area of Asia and sub-Saharan Africa, and more recently in the Pacific, the Caribbean, and South America (Fig. 10), while serologic evidence of infection also has been found in Pakistan, Saudi Arabia, and Iraq. Historical accounts suggest that CHIKV also may have been brought to the western hemisphere with the slave trade, as was yellow fever virus, and the name dengue may have originally referred to chikungunya virus infections (139). However, the involvement of other viruses (e.g., MAYV) causing a similar clinical syndrome cannot be excluded. Historically, the virus has been transmitted in the urban human-to-human cycle predominantly by *A. aegypti* mosquitoes, but *A. albopictus* has become a major vector of IOL strains since 2005, and other *Aedes* species have been implicated in other outbreaks. *A. albopictus* appears to have been the important vector in the

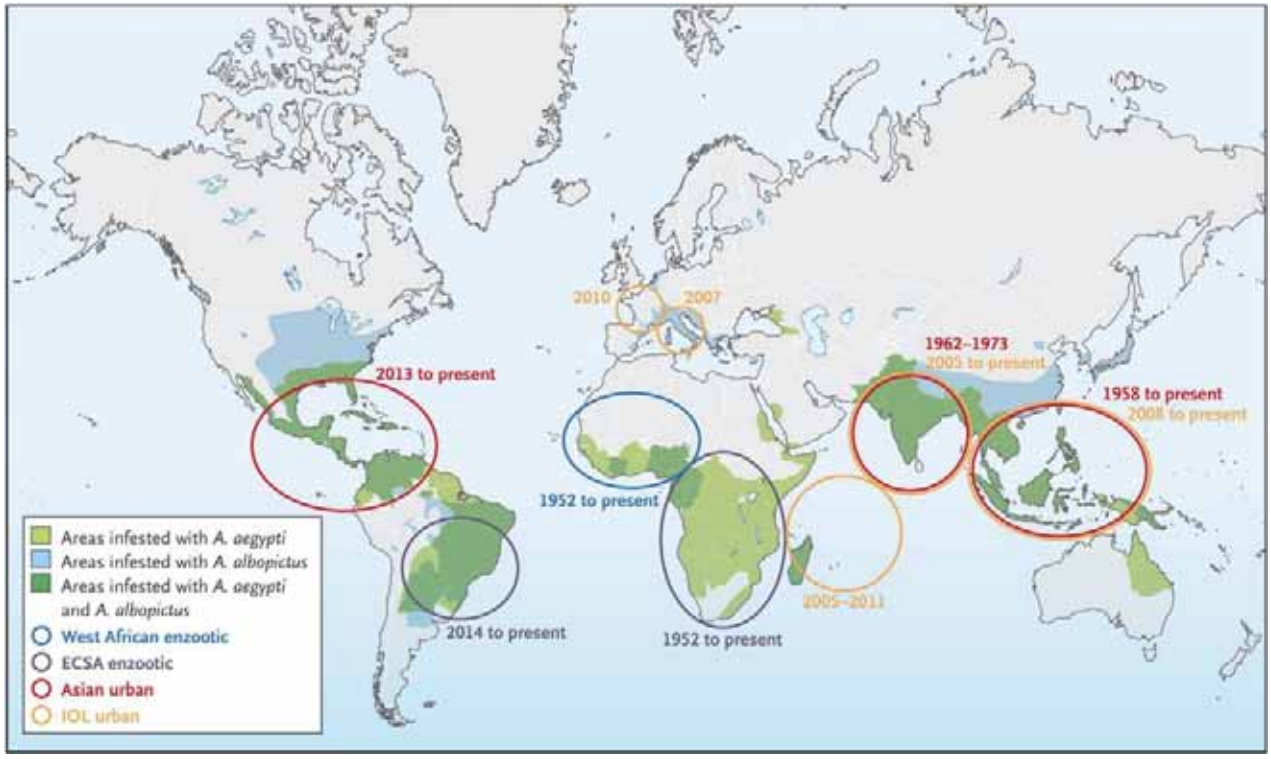

FIGURE 10 Origin, spread, and distribution of chikungunya virus and its vectors showing the African origins of enzootic chikungunya virus strains and the patterns of emergence and spread of the Asian lineage and Indian Ocean lineage (IOL) of the virus and the distributions of *Aedes aegypti* and *A. albopictus*. ECSA denotes eastern, central, and southern African. Reprinted from reference 3 with permission.

recent epidemic in the Indian Ocean region (140) and in Italy in 2007 (141).

(ii) Incidence and Prevalence of Human Infections

Seroprevalence rates of 30 to 100% have been found in enzootic regions of Africa. Sporadic human infections occur with exposure to the enzootic transmission due to occupational activities or travel to nearby villages, with *A. furcifer* implicated in the invasion of villages near enzootic arboreal foci (142). Transmission occurs mainly in the rainy season, when mosquito numbers increase.

(iii) Epidemic Patterns

Extensive epidemics were reported between 1952 and 1977 in eastern and southern Africa, with polyarthritis attack rates of 50% and village-to-village spread occurring in 2- to 3-week intervals. Past epidemics have been infrequent in Africa, but recently several have been documented, some mediated by *A. albopictus* transmission.

In Asia, explosive *A. aegypti*–borne outbreaks have occurred mainly in urban locations where peridomestic breeding sites in discarded containers and water storage reservoirs are prevalent. An estimated 400,000 cases occurred during an outbreak in Madras, India, in 1964, and in Bangkok, Thailand, 40,000 to 70,000 outpatient pediatric cases were seen in 1962 (10). By 1988 the disease had disappeared from the city, but in a sudden reversal, scattered outbreaks in Thailand reemerged without warning in 1995. A similar disappearance from Calcutta, India, was demonstrated in a 1994 serosurvey that showed a 12% antibody prevalence in persons >50 years old but no evidence of infection in children or young adults. Malaysia experienced spread of the virus in 1998–1999, and CHIKV outbreaks also occurred in Indonesia in 1982 and again in 2001 to 2003.

The most recent CHIKV epidemic, involving millions of cases, began in Kenya in late 2004, spread to the Comoros in early 2005, and subsequently spread to the Seychelles, Réunion, Mayotte, and Mauritius. In 2006, the same Indian Ocean lineage strain spread independently from Africa to India, predominantly to the western coastal provinces, and Sri Lanka (140). Attack rates have approached or exceeded 50% of the population in many areas. It also spread to Italy in 2007, the first entry of CHIKV into Europe, followed by Southern France in 2010. Additional spread into Southeast Asia was also accompanied by major outbreaks. Transmission in most of these regions has been predominantly due to *A. albopictus* in the Indian Ocean and Italy (33, 140, 141), augmented by point mutations in the E1 and E2 protein genes that increase the efficiency for transmission by *A. albopictus* (33). Therefore, there was concern that this IOL strain would continue spreading internationally, including to Europe and North America, due to the wide distribution of *A. albopictus* there.

It was therefore a surprise that, when CHIKV circulation in the Americas was finally detected in late 2013, it involved the older Asian epidemic strain that had circulated in Southeast Asia at least since the 1950s (10), suggesting that transmission would mainly be via *A. aegypti*. Initial cases were diagnosed on the Caribbean Island of St. Martin, but the outbreak quickly spread throughout the Caribbean, Central America, northern South America, and into Mexico and Florida in North America. To March 2016, 991,134 cases in the Americas have been reported to the Pan American Health Organization/WHO (143), although the difficulty in distinguishing clinically between dengue and CHIKV, as well as the lack of available diagnostics in many affected regions, raises questions about the accuracy of these estimates.

(iv) Transmission

In Africa, reduction of human infections in areas of previous activity parallels the natural transmission cycle of infections among forest monkeys and sylvatic *Aedes* mosquitoes, in which the virus circulates locally in 5- to 7-year cycles, probably determined by the availability of susceptible amplifying hosts. The virus is presumably maintained by the continuous movement of epizootics over large regions, refreshed by the birth of new cohorts of susceptible animals. *A. aegypti*– *and A. albopictus*–borne outbreaks also have occurred in Africa (including the former in the Tanganyika outbreak that led to the initial recognition of the disease), fueled by the prevalence of peridomestic mosquito breeding sites and, in arid areas, by receptacles of stored water.

Little is known about possible enzootic transmission cycles in Asia, although seropositive monkeys have been found, indicating the possibility of a similar forest primate-mosquito cycle. *A. albopictus* and *Aedes vittatus* are capable of transmitting the virus and are prevalent in areas where outbreaks have occurred, and the former has been shown to be important in the Indian Ocean and European outbreaks (33, 140, 141).

Cases have been reported in travelers and expatriates in tropical locations. CHIKV is also highly contagious in the laboratory; at least 39 cases, due to percutaneous and nonpercutaneous exposures, have been reported. Laboratory manipulations of live virus should be confined to BSL-3 facilities (51).

Clinical Manifestations

In contrast to many other arboviral diseases, the apparent: inapparent ratio for CHIKV infection is high, with generally less than 15% of patients showing evidence of asymptomatic infection (10). The incubation period is estimated to be 2 to 10 days prior to the onset of signs and symptoms, which usually coincide with viremia. The onset of malaise, fever, and joint pains is sudden and typically without prodromal symptoms and is rapidly incapacitating (43). Arthralgia is usually symmetrical, involving the knees, elbows, fingers, feet, ankles, and, less often, the shoulders and hips (47, 144). Backache and headache are common. Inflammation of the fascia of the sole of the foot and the wrist is often reported, and pressure on nerves due to swelling of the fascia may cause tingling in the extremities.

Patients appear to be distressed, holding their extremities motionless. In children, high fevers (>40°C) are typical, but fever may be entirely absent in adults. The conjunctivae are suffused, and the pharynx may be mildly inflamed. Rarely, the ear pinnae may be painful and inflamed (43, 145). Lymph nodes may be slightly enlarged and tender. Affected joints are warm and painful to palpation and exhibit periarticular fullness; definite effusions are present in about 10% of cases. Stiffness and intense pain accompany movement. Rash occurs in as many as 50% of cases, appearing either with the onset of symptoms or several days later, with the decline of fever. It is a faint, irritating, or pruritic maculopapular rash initially on the trunk and face and spreading to the extremities, including the palms and soles. Patients may develop fine petechiae, and occasional purpura may appear on the trunk and limbs, especially on the legs and feet, with or without gingival bleeding. The liver may be slightly enlarged, although not as prominently as in dengue hemorrhagic fever.

After 2 to 4 days of illness, improvement is often rapid, while joint pain and stiffness resolve more slowly and can persist for months or years in some cases (see below). Arthritic symptoms consist of morning stiffness, pain, and swelling of the joints, impairing function. Fever may be biphasic in children. The rash also may reappear in 3 to 7 days, with as many as three successive eruptions. CNS involvement, consisting of meningismus, nuchal rigidity, ophthalmoplegia, slurred speech, and limb weakness, has been described in individual cases. During the Réunion Island epidemic, CHIKV-associated encephalitis occurred at a rate of 8.6 per 100,000 patients, with the highest ratios in infants less than 1 year old (187 per 100,000) and in adults more than 65 years old (37 per 100,000) (146). Persistent convulsions associated with neurologic sequelae have been reported for infants, who are at high risk of infection (up to 50%) during birth if the mother is viremic (10, 145, 147). Acute polyneuropathy and paralysis have also been described (147).

(i) Outcomes and Complications

Myocarditis, hepatitis, and multi-organ failure may occur in severe cases, but hemorrhagic phenomena are rare. During recent outbreaks, some fatalities associated with CHIKV infection have been noted, especially in newborn and elderly populations, frequently associated with neurologic disease. The overall case-fatality rate averages about 0.1% (10). Severe and fatal cases of CHIKV infection often manifest with high fever, prolonged convulsions, and neurologic deficits suggesting primary encephalitis (145–147). The 2005 Indian Ocean outbreak of CHIKV infection was associated with a much higher incidence of serious disease than previously described (140). However, many of these cases involved other underlying illnesses that contributed to the poor outcomes or followed peripartum transmission from symptomatic mothers resulting in severe disease in the neonates (10, 145, 148). Ocular involvement, including uveitis and optic neuritis, may occur. Older patients and those with comorbidities, such as cardiovascular, neurologic, respiratory disorders, or diabetes, are at higher risk for severe CHIKV disease requiring hospitalization. Early nasal skin necrosis was reported in three patients with severe CHIKV disease in Venezuela in 2014, and involvement of the nasal skin has been reported in previous outbreaks (149).

CHIKV-induced chronic polyarthralgia and/or polyarthritis can be persistent or relapsing over many weeks or months (43) and causes substantial incapacitation in as many as 50% of patients. It involves mainly the distal joints, mimicking rheumatoid arthritis, and may manifest as chronic inflammatory, erosive, and, rarely, deforming polyarthritis. Children and young adults typically have mild transitory joint pains, while arthritic symptoms are substantially more severe and persistent in older people. Morning stiffness and other symptoms typically persist for several weeks or months and, in a small percentage of cases, continue for years (43). A 3- to 5-year follow-up of 107 patients in South Africa found that one-third had fully recovered within several weeks; one-third had a slower resolution, over about a year; and in 14%, recovery took 2 to 3 years (150). Fully recovered patients were younger, with a mean age of 37 years. Residual symptoms ranged from mild discomfort and stiffness to persistent pain with effusions or synovial thickening but no destructive changes or muscular atrophy. Joint fluids in three cases showed an elevated WBC count (2×10^9 to 5×10^9/liter) and, in one case, reduced complement levels (151). Low titers of rheumatoid factor (mean, 1:2), indicative of low-grade inflammation, and elevated ESRs (15 to 25 mm/h) have been reported for patients with persistent arthritic symptoms. Radiographs have shown mainly soft tissue swelling, but small erosions were noted in a metacarpophalangeal joint of one patient (151).

(ii) Laboratory Abnormalities

The peripheral WBC count usually is normal or slightly depressed, whereas children may present with a neutrophilia followed by a relative lymphocytosis (43, 145, 146). Markedly depressed platelet counts, $< 50 \times 10^9$/liter, have been observed in patients with hemorrhagic manifestations. Prothrombin and bleeding times have been normal in a few reported cases. The erythrocyte sedimentation rate (ESR) may be increased to 20 to 50 mm/h, with elevated C-reactive protein. Various joint fluid abnormalities have been described in cases with persistent arthritic symptoms (see below).

In patients with neurological diseases, the CSF protein and leukocyte count are usually elevated, especially in those with encephalitis (147). Routine electrocardiographs have disclosed changes suggestive of myocarditis in three cases (148).

(iii) Differential Diagnosis

In endemic or epidemic regions, the debilitating polyarthralgia has a positive predictive value as high as 80% for CHIKV viremia in adults and children (10). Dengue is the principal consideration in the differential diagnosis because of its overlapping geographic distribution, vectors, and transmission season and its similar clinical presentation, with acute fever and musculoskeletal pain. CHIKV produces a more severe and immediately debilitating illness, with prominent polyarthralgia. Dengue is characterized by more severe constitutional symptoms, retro-orbital headache, and eye pain and is less likely to produce a clinically apparent rash, and if it does, the rash differs from the maculopapular rash seen with CHIKV infection. Principal laboratory findings of CHIKV include lymphopenia, often closely associated with viremia, thrombocytopenia, increased levels of aspartate aminotransferase and alanine aminotransferase in blood, and hypocalcemia. Thrombocytopenia is also relatively common in dengue. The illnesses are less easily differentiated in children, who have milder joint symptoms than do adults with CHIKV infection. ONNV illness is very similar but with more prominent cervical lymphadenitis and conjunctivitis. SINV disease also occurs in southern Africa and causes polyarthralgia and rash, though with a milder illness and less fever than that due to CHIKV. In travelers returning to areas with endemic arboviruses, such as RRV and BFV in Australia and SINV in Scandinavia, the illness may resemble locally acquired infection. In addition, rheumatic symptoms may be associated with various parasitic infections endemic in Africa or Asia. The differential diagnosis also should include cosmopolitan infections due to viruses producing acute polyarthritis, including parvovirus B19 and rubella virus, as well as hepatitis B and C viruses, human immunodeficiency virus, mumps virus, enteroviruses, and Epstein-Barr virus, and post- and para-infectious arthritis due to acute rheumatic fever, bacterial and parasitic enteric infections, Reiter's syndrome, and disseminated gonococcal infection. Serum sickness and Henoch-Schönlein purpura also should be considered in the diagnosis. Cases with acute arthritis involving small joints of the hands, wrists, knees, and elbows can mimic acute

rheumatoid arthritis, and the chronicity of symptoms in some cases adds to the difficulty of making the appropriate diagnosis.

Laboratory Diagnosis

High and relatively persistent levels of viremia have been observed ($>10^{8.0}$ PFU/ml), peaking on the day of onset of fever, declining to approximately $10^{1.0}$ PFU/ml by the fifth day. Viral RNA can often be detected in blood within 1 week of onset and may persist longer (152, 153). Viral RNA has been detected in the CSF of patients with neurological disease, more often in encephalitic infants than adults (146). It has not yet been found in the joint fluids or blood of patients with persistent symptoms, but testing has been limited.

Most diagnoses are serologic, using hemagglutination inhibition (HI) or immunofluorescence (IF) assays or, most commonly, enzyme immunoassay (EIA) (154, 155). A number of commercial and in-house assays have been used, with variable performance on quality assurance programs (155). Indirect and isotype-capture EIAs for virus-specific IgM are positive within the first few days of illness and generally indicate recent infection. However, IgM persists for several weeks or months and may indicate recent past infection. A ≥4-fold rise in IgG titer or IgG seroconversion supports recent infection. On Réunion Island, IgM was detected in the CSF of 21/52 patients with CHIKV neurological disease, including 10/15 adult encephalitis cases but in none of the encephalitic infants (146).

Cross-reacting antibodies are uncommon but may occur due to ONNV and possibly other alphaviruses, and where potential exposure to more than one arthritogenic virus has occurred, IgM and IgG tests should be carried out for all. If there is IgM reactivity to more than one virus, then neutralization tests are usually required to determine the infecting virus.

Prevention

Outbreaks of *A. aegypti*– or *A. albopictus*–borne diseases are best prevented by destroying or removing containers holding water that serve as breeding sites, following the approaches described for dengue control. *A. albopictus* utilizes a wider range of larval habitats, so control cannot rely only on peridomestic environments. Personal protective measures against mosquito bites are indicated to prevent exposure to these urban vectors, including during the daytime when they typically bite and remain in houses, as well as to reduce exposure in sylvatic settings, which also involve nighttime biting mosquitoes. Newer investigational strategies for vector control include the release of transgenic *A. aegypti* engineered to carry a late-acting lethal transgene (156) and the use of Wolbachia bacteria, which reduce the competence of both urban vectors for transmitting CHIKV and DENV.

Treatment

Symptomatic treatment with nonsteroidal anti-inflammatory drugs provides relief from joint stiffness and pain in many patients, though individuals respond variably to the different drugs. Rest, heat, and gentle exercise may assist, but vigorous exercise should be avoided. There are no specific antiviral agents for any of the alphaviruses or specific therapies for alphavirus-induced arthritis. A number of compounds have shown activity against chikungunya *in vitro*, and two of these have shown effectiveness in mice: favipiravir (T-705) protected against arthritis and viremia, while an anti-E2 monoclonal antibody was effective in treatment and prophylaxis in mice (157). Severe arthritis may be managed with immunosuppressive therapies used for RA, but this should be done with caution.

O'NYONG-NYONG VIRUS

The name of ONNV derives from the Acholi term meaning "very painful and weak," which was given to describe the illness in the first recorded outbreak (158). The epidemic, resulting in an estimated 2 million cases, emerged in 1959 in Uganda and spread rapidly in East Africa, south to Mozambique, and west to Zaire, and to Senegal in West Africa before dying out 3 years later. Another outbreak occurred in the Ivory Coast in the mid-1980s, but the virus did not reappear in East Africa until another major epidemic erupted in 1996–1997, leading to an extensive outbreak in southern Uganda (159, 160). In retrospect, the virus had been circulating locally at a low level, and virus recovered from those patients and the 1996 and 1959 strains proved to be similar genetically (161; R. Swanepoel, E. Sanders, and T. Tsai, unpublished data). Most recently the virus has been found in Chad (162).

As previous field studies indicated, *Anopheles funestus* was implicated as the principal mosquito vector in outbreaks and, interestingly, ONNV is the only virus known to use anophiline mosquitoes as its major vector. Adaptation of the virus to peridomestic *Anopheles* mosquitoes undoubtedly contributes to the explosive nature of its epidemic transmission (163). The molecular determinants of this unique vector specificity appear to reside in the nonstructural protein 3 (nsP3) gene (164). Other aspects of the viral transmission cycle have not been elucidated, but the presence of an unidentified nonhuman mammalian host may account for the maintenance of the virus between epidemics.

Clinically, the illness resembles that due to CHIKV and the other arthritogenic alphaviruses and is characterized by fever, constitutional symptoms, joint pain, rash, and lymphadenitis, the full syndrome appearing in 40% of cases. Differentiation may be difficult; however, lymphadenopathy, occurring in 50% of cases, seems to be more marked in ONNV disease (159).

Virus can be recovered from acute-phase blood samples taken within the first 6 days after onset, and virus recovery may be successful in afebrile patients (159, 160). Laboratory diagnosis is usually done by serology, as discussed for CHIKV.

Personal protective measures against malaria should also be effective against acquiring ONNV, i.e., using mosquito nets impregnated with permethrin and avoiding unprotected evening and nighttime exposure.

Igbo-Ora virus, a genetic variant of ONNV (161), was isolated in 1966 from blood samples of febrile patients in Igbo-Ora and Ibadan, Nigeria, and cases subsequently were reported from the Central African Republic and the Ivory Coast. The clinical illness, described in only one case, consisted of fever, polyarthritis, and pharyngitis.

SINDBIS VIRUS

SINV, named after the northern Egyptian district where the virus was first isolated in 1952, is transmitted in Europe, Africa, Asia, and Australia and is thus the most widely occurring alphavirus. Strains are separated into Europe/Africa and Asia/Australia lineages. The close genetic relationship of northern European and South African strains suggests that they were originally introduced from South Africa to Scandinavia by migratory birds (165). While Australian

SINV is predominantly the Asia/Australia lineage, a third lineage has been identified in the southwest of Western Australia that shows considerable genetic divergence from the South African and European strains and that appears to be restricted in its distribution (166). Novel outbreaks, occurring in 1981 in Sweden, Finland, and the adjacent Karelia area of Russia, were given local names: Ockelbo, Pogosta, and Karelian fevers, respectively. Subsequently, endemic transmission was recognized in those locales and in Norway.

Epidemiology

In Africa, sporadic cases and occasional outbreaks numbering in hundreds or thousands of cases were first reported in 1954 to 1956, principally from areas of South Africa during the summer from December to April (167), but it is an uncommon illness in travelers returning from that country.

In Sweden, 600 to 1,200 cases are estimated to occur annually (168, 169), while Pogosta disease recurs in a 7-year cycle. Transmission is localized to a zone between latitudes 60° and 63° N, with declining incidence and seroprevalence north and south of this zone. Most cases occur from July to September during the season of most active viral transmission among middle-aged adults with woodland exposure (e.g., while picking berries or gathering mushrooms).

In contrast, SINV rarely causes human disease in Asia or Australia, despite the fact that serologic studies in Australia have shown evidence of regular human infections. This suggests that there may be pathogenicity differences between the different lineages (170).

The maintenance cycle for SINV is thought to be a mosquito-bird cycle. Humans are dead-end hosts infected by the bite of ornithophilic mosquito species or by bridging mosquito vectors with broader feeding habits. These include *Aedes*, *Culex*, *Culiseta*, and *Mansonia* spp.

Clinical Manifestations

The incubation period is as long as 1 week, and the principal clinical features are arthralgia and rash (167–169). Symptoms may be preceded by a short prodrome of fever, headache, and malaise followed by progressive musculoskeletal pain. All the joints may be symmetrically involved, although the ankles, wrists, knees, fingers, and toes are most frequently affected, followed by the hips, shoulders, elbows, and, occasionally, the neck and back. Joints are swollen due to synovial and periarticular edema, and some patients are unable to walk. Tendons may be inflamed as well. Pharyngitis and lymphadenopathy may be present. Discrete macules on the trunk and limbs, including the palms and soles, evolve to small (3 mm diameter) papules, generally sparing the face and head (Fig. 11), and a vesicular rash occasionally develops. The ESR may be elevated during the acute illness, occasionally to >25 mm/h. Other laboratory examinations have been unremarkable.

Joint symptoms usually resolve over a period of weeks, but residual symptoms persist for several years in one-third of cases (49). Convalescence is characterized by asthenia and fatigue.

The principal consideration in the differential diagnosis is West Nile virus infection, which is transmitted in the same-season enzootic cycle and in an overlapping geographic distribution in Africa and Asia. However, West Nile virus causes less prominent joint pains, rash is less common, and lymphadenopathy may be more prominent. Other conditions to be differentiated are described in the section on CHIKV above.

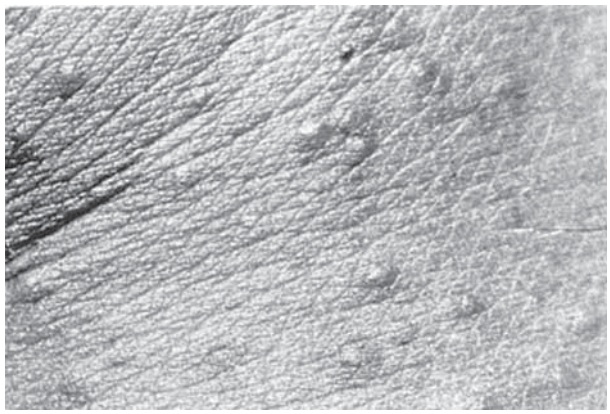

FIGURE 11 Small papular lesions seen in Sindbis (Ockelbo) fever. Courtesy of B. Niklasson.

Laboratory Diagnosis

Virus has been recovered from both blood and skin lesions of a minority of infected individuals but has limited diagnostic value (171). Specific IgM generally appears within a week after onset, and IgG appears a few days later (171). IgM declines slowly over a period of years (172). Cross-reacting antibody has not been described as a problem, and it should be considered where exposure to other alphaviruses may have occurred.

Prevention and Treatment

Individuals planning excursions to known enzootic areas during the transmission season should use precautions against mosquito bites. No specific therapy is available. Symptomatic treatment with nonsteroidal anti-inflammatory drugs may provide relief from joint symptoms.

MAYARO VIRUS

MAYV is a member of the SFV complex of alphaviruses and is its principal representative of alphaviruses within the New World. Viral activity is widely distributed in forested locations in Central America and the pan-Amazonia region of South America, resulting in a high level of endemic transmission and seroprevalence rates of >50% in some areas. The virus was isolated in 1954 from febrile humans in scattered areas of Trinidad and was named after the island's Mayaro District (30, 173). Phylogenetic studies have shown that MAYV exists in two distinct genotypes; genotype D contains isolates from Trinidad and the north central portion of South America, including Peru, French Guiana, Surinam, Brazil, and Bolivia, whereas genotype L appears to be restricted to the Amazonian region of Brazil (173).

Most outbreaks are typified by forest exposure, high attack rates, often with higher risk among males of working age, and occurrence in the rainy season. Outbreaks in cities have also been described. The epidemiological pattern is explained by the forest cycle of viral transmission, probably between *Hemagogus* mosquitoes and wild vertebrates, including monkeys and marmosets, analogous to the sylvatic cycle of yellow fever.

The incubation period is <6 to 12 days (174, 175). Onset of illness is sudden, with severe headache, vertigo, chills, myalgia, malaise, and fever as high as 40°C. Movement of the eyes, head, and neck is painful, and the conjunctivae may be injected. Joint pain and swelling are the

principal features of the illness, affecting (in decreasing frequency) the fingers, wrists, ankles, toes, elbows, and knees. Joint pains sometimes precede the onset of fever and range in severity from mild to incapacitating. In two-thirds of cases, a morbilliform rash over the trunk and limbs appears late in the illness, often with the resolution of fever. Rash occurs more often in children than in adults. Inguinal lymphadenopathy is present in about half the cases. Slight liver enlargement and mild jaundice have been reported. Fever remits after 1 to 6 days, and patients with mild symptoms are able to resume work in 2 to 3 days. Others remain incapacitated with fatigue and joint stiffness for several weeks. Leukopenia with a relative lymphocytosis is a constant finding in the first week, with values returning to normal in the second week. Moderate albuminuria and slight elevations of liver transaminases and direct bilirubin have been reported for a few patients (174). Hemorrhagic disease has been described, but no fatalities have been attributed to the illness.

Clinically the illness may be mistaken for dengue. An epidemiological history of forest exposure suggests the diagnosis, and other considerations are discussed in the section on CHIKV above.

The virus can readily be isolated from blood obtained within the first 3 or 4 days of illness (30, 174, 175). Detection of specific IgM and detection of a ≥4-fold or greater rise in IgG titer provides a serologic diagnosis of recent infection, but reciprocal testing with antigens against other local alphaviruses may be needed (175).

Infection is best prevented by avoiding forested areas, particularly during the day when *Hemagogus* mosquitoes are active, and by taking appropriate measures to prevent exposure.

ROSS RIVER VIRUS

Virology

RRV is the most common arbovirus infection in Australia. Outbreaks of "epidemic polyarthritis" infection were recognized in 1886 and 1928 and also during World War II before the virus was isolated in 1959 from *A. vigilax* mosquitoes collected along the Ross River in Queensland (8). The virus exhibits a high degree of conservation, with strains from the known region of viral transmission, spanning a 30-year period, showing only a 3.3% divergence in the nucleotide sequences of the E2 and E3 genes. Strains separate into three genotypes that have demonstrated strain replacement (genotype 2) and extinction (genotype 1). Genotype 1 was present in northern Queensland until the mid-1970s when it was replaced by genotype 3, which also caused the outbreaks in the Pacific Islands. Genotype 2 is restricted to the southwestern corner of Western Australia but was largely replaced by genotype 3 in 1996, which is now the dominant strain throughout Australia (170). The reasons for this genotype 3 dominance may be due to a fitness advantage related to duplications of 36 nucleotides (and their resulting polypeptide) in the 3′ region of the nsP3 gene (176). Complete genome sequences are available for genotype 1 (T48) and several genotype 3 strains, including one human isolate (QML 1), and a full-length cDNA clone of the T48 strain has been constructed.

Epidemiology

Cases have been reported from all states of Australia, Papua New Guinea, New Caledonia, the Solomon Islands, and, in a single extensive outbreak in 1979–1980, from Fiji, American Samoa, Tonga, and the Cook Islands in the South Pacific (170). Several thousand cases of RRV disease are reported each year in Australia, the majority occurring in the heavily populated areas of southeastern Queensland, coastal New South Wales, and the southwest region of Western Australia, although the highest attack rates and individual risk actually occur in the sparsely populated northern tropical areas (Fig. 12). In the subtropical and tropical northern regions, human infections occur mainly in the December-to-May wet season, and, in the central arid regions, outbreaks occur following irregular heavy rainfall and flooding. In the southerly temperate climates, infection is highest in the late spring, early summer, and autumn, when it is warm and wet (177). Risk is directly related to proximity to mosquito breeding areas (56). A low level of viral transmission may occur throughout the year, even in temperate southern Australia. Epidemics in these temperate areas generally occur every 2 to 4 years and are likely related to the climatic conditions resulting in increases in vector numbers and to the availability of susceptible amplifying marsupial hosts (178).

The principal vectors are salt marsh *Aedes* species, especially *A. vigilax* in coastal areas, and *Culex annulirostris* and other freshwater species in the interior (170, 177). Natural infections occur in a broad range of animals, including birds, marsupials, and placental mammals. The principal viral transmission cycle in Australia involves kangaroos, wallabies, and other macropods, especially younger animals (178), as vertebrate hosts, with humans as incidental hosts. Short cycles of human-mosquito-human transmission have occurred during major epidemics (170) but are not sustained and do not significantly contribute to maintenance of the virus.

Epidemics typically have been preceded by increased rainfall and increased tidal inundation of coastal swamps, leading to expanded vector mosquito populations. Risk is associated with outdoor exposure during the periods of greatest mosquito activity, during the day and at night for *A. vigilax*, and in the period before and after dusk and dawn for *C. annulirostris*. The highest rates of infection occur in residents of epidemic areas, but infections in tourists and other visitors are not unusual (177). Recently, transmission by blood transfusion has been documented (179), adding to the list of transfusion-transmitted arboviruses.

Clinical Manifestations

The estimates of clinical illness following RRV infection have varied widely, with case-to-infection ratios between 1:80 and 3:1 or higher (36, 43, 48). The incubation period can last 3 to 21 days, but usually ranges from 7 to 9 days (48). The acute illness consists of malaise, fatigue, muscle pains, joint pains, and, in one-half of cases, low-grade fever (177, 180). Some patients have diarrhea, headache, neck stiffness, or sore throat. Within a few days, a maculopapular erythematous rash appears in 50 to 60% of cases, initially on the trunk and limbs and sometimes spreading to the palms, soles, and face. It may appear as early as 11 days before or as many as 15 days after onset. Occasionally the rash is predominantly papular and, rarely, vesicular. Enanthems of the oral mucosa are uncommon. The rash generally fades or desquamates within 10 days, although lesions may recur. Tiredness, debilitating joint pain, stiffness, and swelling develop in 80 to 90% of patients; joint involvement is usually symmetric and typically affects the wrists, knees, ankles, and small joints of the hands and fingers. The elbows, shoulders, feet, back, and jaw may also be involved and, less frequently, the hips and costochondral junctions. Inflammation may

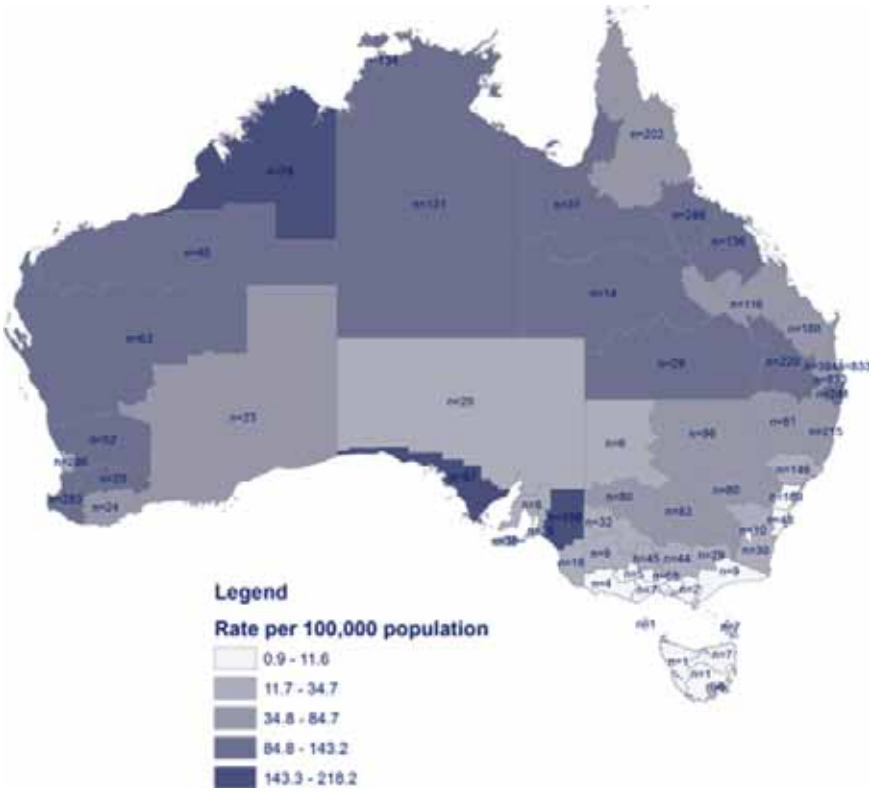

FIGURE 12 Notifications and notification rates of RRV infections, Australia, 2005–2006, by Statistical Division of residence. Reprinted from reference 187 with permission.

extend to wrist and ankle tendons and the plantar fascia, causing nerve compression and paresthesia.

Most patients recover within a month, with joint pain, lethargy, and muscle pains being the slowest symptoms to resolve. Fever and rash, if present, usually last less than a week; many patients recover fully within 4 weeks, and most return to full physical activity within 3 to 6 months (177, 180), although symptoms, such as joint pains, muscle pains, and lethargy, may continue for months, especially in patients with preexisting joint disease (42, 43).

Glomerulonephritis and associated loin pain have been noted in several cases. Vertical infections have been demonstrated in experimentally infected mice and may occur rarely in humans, but there is no evidence of any associated fetal disease in humans. While headache is relatively common, there is no convincing evidence of more serious neurologic disease due to RRV (177).

The blood count is nearly always normal, and the C-reactive protein level is rarely elevated. Rheumatoid factor and other autoantibodies are absent. Cases with glomerulonephritis may have hematuria with the presence of glomerular red cells, pyuria, and proteinuria. The ESR may be moderately elevated during the first week of illness and usually declines to normal despite the persistence of symptoms. Joints, even if swollen, often have little excess synovial fluid, although amounts as high as 70 ml have been reported. The joint fluid is clear, opalescent, and free of clots and exhibits a mononuclear pleocytosis of 1×10^9 to 60×10^9 cells/liter. Radiographs show no erosive changes or deformities.

The differential diagnosis includes other arthritogenic arboviruses found in Australia, e.g., BFV, two flaviviruses (Kokobera virus and the Kunjin strain of West Nile virus), and, rarely, SINV (170, 180). Other considerations in the differential diagnosis are mentioned in the section on CHIKV.

Laboratory Diagnosis

Humans have a transient viremia, so virus is only occasionally detected in acute-phase blood. Culture is usually done in C6/36 cells, although blind passage to indicator cells, such as Vero, BHK, or chicken embryo cells, is needed to see a CPE. Intrathoracic injection of *Toxorhynchites* mosquitoes or inoculation of mice is less common. Detection of viral RNA is more sensitive than culture and may be positive prior to, and in the early phase of, the antibody response. However, viral RNA is usually undetectable by the time patients present to a medical practitioner, and that, combined with the limited availability, means that it is not used in routine diagnosis.

Laboratory diagnosis is usually made serologically. Confirmed recent infection is indicated either by IgG seroconversion by EIA, HI, or NT or by a 4-fold rise in IgG titer by HI or NT. This may take as long as 4 weeks and occasionally more. Virus-specific IgM antibodies, detectable by EIA or IF, appear within 1 week of onset of illness. False-positive reactions can occur such that detection of IgM alone, especially using some of the commercially available EIAs, is likely to be a false-positive result and should always be confirmed by convalescent serology to check for IgG seroconversion (54). Cross-reactions between RRV and BFV antibodies are uncommon and usually occur with EIA IgM tests; the infecting virus can usually be determined by looking for rising IgG or retesting using a different assay.

Prevention and Treatment

Mosquito control measures can be undertaken to control breeding in areas near human populations. Personal protective measures are recommended for people living in or visiting areas of RRV activity. There are no vaccines available. An inactivated vaccine for Ross River virus has been shown to be safe and immunogenic in humans, inducing neutralizing antibody responses in 91.5% of adults younger than 60 years and in 76% of adults aged 60 years or older, following three doses (181). However, as the vaccine has never been licensed for use, its effectiveness in prevention of infection and disease in humans is not known.

Rest during the acute period of illness is prudent, followed by gentle exercise. Heat assists some patients. Nonsteroidal anti-inflammatory drugs provide rapid relief of symptoms and hasten resumption of activities in most patients (48). Steroids have been used and provide temporary relief, but they are not recommended due to potential side effects and a lack of data on benefits.

BARMAH FOREST VIRUS

BFV, named after the site in northern Victoria where it was first isolated from *Culex annulirostris* mosquitoes, is antigenically distinct from other alphaviruses, including RRV and SINV, which are also found in Australia (170, 177). The diseases caused by BFV and RV are clinically indistinguishable, although arthritis and arthralgia are less severe, occur less frequently, and do not persist as long as with RRV disease, and rash is more prominent in BFV disease (180). In a mouse model, BFV caused less severe myositis than did RRV and showed lesser activation of inflammatory mediators TNF-α, IL-6, CCL2, and arginase-1 than did RRV (182).

Their seasonality and transmission cycles have overlapping features, but independent outbreaks have occurred. Overall, the BFV notification rates in Australia are usually 5 to 10% of those for RRV disease (177). Apparent increases in infections in recent years have resulted from an increase in testing and from high false-positive rates in the commonly used EIA-IgM test (55). Patient management is the same as for RRV disease, and there is no vaccine available.

MISCELLANEOUS ALPHAVIRUSES

Semliki Forest Virus

In Central Africa, SFV is transmitted in a sylvatic cycle analogous to that of yellow fever virus (183). While human infections are common, disease is rare, with one case of febrile illness with severe persistent headache following natural infection in Africa and one laboratory-acquired case of fatal encephalitis in Germany.

Me Tri virus is a variant of SFV (184), which was isolated from *Culex tritaeniorhynchus*, and has been associated with sporadic encephalitis cases in children in Vietnam (185).

Getah Virus

GETV is distributed widely in Asia and Oceania. It causes disease in horses and abortion in pigs and is a rare cause of febrile illness in humans.

Una Virus

UNAV is closely related to Mayaro virus, but phylogenetic studies show that it is quite distinct genetically. It produces febrile illness with arthritis in horses but has not been associated with human disease.

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Rubella Virus

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Rubella is a benign disease when acquired by a child or adult, but causes significant sequelae to a developing fetus when intrauterine transmission occurs. Following the devastating worldwide pandemic in 1962–1965, a safe and effective rubella vaccine was developed and widely utilized. As a consequence, the occurrence of congenital rubella syndrome has decreased dramatically in those regions of the world with rubella vaccination programs. In 2015 the Americas region became the first World Health Organization (WHO) region in the world to be declared free of endemic transmission of rubella. Despite this, rubella continues to circulate in other parts of the world, including a large outbreak in Japan occurring since 2012, and approximately 100,000 cases of congenital rubella syndrome still occur worldwide. Given the ease and frequency of global travel, however, clinicians need to remain aware of rubella even in the United States, so that imported cases can be identified and managed accordingly. This chapter reviews the current knowledge of the natural history, pathogenesis, diagnosis, treatment, and prevention of rubella.

INTRODUCTION

Rubella was known to early Arabian physicians by the name *al-hamikah*, but it was initially considered to be a form of measles (1). In 1752 and 1758, the German physicians de Bergen and Orlow first described rubella as a unique clinical entity (2, 3). Rubella subsequently was reported in England (3–5) and the United States (3, 6). In 1866, Henry Veale introduced the name *rubella*, believing that the name of a disease "should be short for the sake of convenience in writing, and euphonious for ease in pronunciation" (7). At the International Congress of Medicine in London, England, in 1881, these developments culminated in the consensus that rubella was a distinct disease (8, 9). Rubella is commonly called German measles, and it is the third of the six viral exanthems of childhood, with measles and scarlet fever being first and second, respectively (10).

Rubella was thought to be a benign disease until 1941, when the Australian ophthalmologist Norman McAlister Gregg first described the congenital defects of infants of mothers who had developed rubella early in pregnancy (11, 12). Although initially met with skepticism by the worldwide medical community, Gregg's keen observations were quickly confirmed in Australia (13–15), the United States (16), and the United Kingdom (17). By 1947, 521 cases of congenital rubella had been reported in the medical literature (18).

In 1938, Hiro and Tasaka (19) established the viral etiology and transmissibility of rubella by subcutaneously inoculating 16 nonimmune children with filtered nasopharyngeal saline washings collected from patients in the eruptive stage of rubella. Habel (20) used similar nasal washings obtained within 24 h of the appearance of the rash to infect *Macaca mulatta* monkeys. Rubella was successfully cultivated in tissue culture in 1962 by Weller and Neva (21) in Boston, and by Parkman et al. (22) in Washington, DC. The methodology of Parkman and colleagues for the isolation of the noncytopathic rubella virus exploited its interference with the growth of enteroviruses in African green monkey kidney (AGMK) cell culture, and this soon became the standard method for rubella virus isolation.

The increasing recognition of congenital rubella syndrome during and after the pandemic of 1962 to 1965 emphasized the need for the development of an efficacious vaccine. Between 1965 and 1967, several attenuated rubella virus strains were developed and evaluated in clinical trials (23–25). Results of these investigations culminated in the convening of the International Conference on Rubella Immunization in February 1969 (26). During the same year, three strains of live attenuated rubella vaccines were licensed in various countries: HPV-77, grown in duck embryos for five passages (DE-5) or dog kidney cells for 12 passages (DK-12); Cendehill, grown in primary rabbit cells; and RA27/3, grown in human diploid fibroblast culture (24, 25, 27, 28). Developed by Stanley Plotkin, the RA27/3 vaccine has been used exclusively in the United States since 1979 (29, 30). Due to the overwhelming success of the rubella immunization program, endemic transmission of rubella has been eliminated in the United States and throughout the Americas (31, 32).

VIROLOGY

Classification

Rubella virus is the sole member of the *Rubivirus* genus of the *Togaviridae* family. The other genus in the family of *Togaviridae* is *Alphavirus*. In contrast to the *Alphaviruses*, which

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replicate in arthropods and in vertebrates, rubella virus has no invertebrate hosts. The only known host for rubella virus is humans.

Only one immunologically distinct serotype of rubella virus exists, and rubella virus is serologically unrelated to other known viruses. At least two genotypes can be distinguished by E1 gene sequences. Rubella genotype I isolates, predominant in Europe, Japan, and the western hemisphere, segregated into discrete subgenotypes. Rubella genotype II viruses are limited to Asia and Europe, demonstrate greater genetic diversity, and may consist of multiple genotypes (33). However, these biological variations are not the consequence of antigenic differences, as determined by protein composition or serologic analysis (34, 35).

Viral Composition

The spherical particles of rubella virus measure 50 to 70 nm in diameter (36, 37). An individual virion is composed of a 30-nm core structure surrounded by a single-layered envelope measuring 10 nm in thickness. The envelope is acquired during budding through the host nuclear or plasma membrane (37, 38). Glycoprotein projections measuring 5 to 6 nm in length are located on the viral surface (38, 39).

The viral core contains the single-stranded, positive-sense 40S RNA genome, composed of approximately 10,000 nucleotides, with a molecular weight of approximately 3.8×10^6 daltons (40, 41). The structural protein known as protein C (or capsid protein) is one of three structural proteins of rubella virus and is associated with the 40S RNA. Protein C is nonglycosylated and has a molecular mass of 33 kDa (39). The other two major structural proteins, E1 and E2, are envelope glycoproteins that together comprise the viral surface projections described above. The molecular masses of E1 and E2 are 58 and 42 to 47 kDa, respectively. E1 is the viral hemagglutinin, and the exact function of E2 is unclear. A 42-kDa E2 molecule (designated E2a) and a 47-kDa E2 molecule (designated E2b) have been recognized (39).

Biology

Viral genomic and subgenomic RNA species are detectable in tissue culture 12 h postinfection, with peak RNA synthesis occurring by 26 h following initial infection (42). In comparison, viral protein production can be initially detected by immunofluorescence at 12 h postinfection, and peak structural protein synthesis occurs by 16 h after initial infection (42).

Propagation of rubella virus in tissue culture does not produce a reliable or distinctive cytopathic effect (CPE) by light microscopy. In general, growth of rubella virus in continuous cell lines (hamster, rabbit, simian, and human) produces a variable CPE which depends upon adaptation of the viral isolate to the cell line and on the passage history of the cell line, among other factors (41). Of the continuous cell lines, the kidney cell lines from the rabbit (RK-13), African green monkey (Vero), and baby hamster (BHK-21) are used most frequently for the detection of CPE.

Detection of rubella virus in primary cell culture (human, simian, bovine, rabbit, canine, or duck) is accomplished by means of an interference assay. Although infection with rubella virus in primary cell cultures does not produce CPE, superinfection by many additional viruses is blocked. AGMK cells have proven superior for isolation of virus from human specimens by the interference technique: infection in AGMK tissue culture by rubella virus is suggested by the failure of the typical enteroviral CPE to occur after challenge with echovirus 11 or other enteroviruses. The presence of rubella virus is then confirmed by an additional technique, such as neutralization or fluorescence with specific anti-rubella serum.

Although natural rubella infection occurs only in humans, infection in experimental animals can be achieved. The complete spectrum of acquired or congenital disease is not manifested in any of these animal models, however. Following intranasal, intramuscular, or intravenous administration, rhesus monkeys develop viremia and shed virus in nasopharyngeal secretions (43). Other species of monkeys can also be infected with rubella virus (44). Attempts at mimicking congenital rubella have resulted in recovery of virus from the amnion and placenta of monkeys, although the embryos are not consistently infected (45, 46). The ferret also has proven to be a very useful animal model in the study of rubella disease following both subcutaneous and intracerebral inoculation of virus (47). Additional animals that have been experimentally infected with rubella virus include rabbits, hamsters, guinea pigs, rats, and suckling mice.

Rubella virus is heat labile. Rapid inactivation occurs at 56°C, and a slower decrease in activity is noted at 37°C (48). In the presence of protein, the virus remains viable at 4°C for a week or more. However, infectivity is rapidly lost at temperatures of −10 to −20°C (48). Specimens can be stored indefinitely at −60°C. The addition of $MgSO_4$ stabilizes the virus with respect to heat inactivation, allowing for safe transport on ice (49). Extremes of pH (less than 6.8 or greater than 8.1), UV light, and chemicals such as ether, acetone, chloroform, deoxycholate, formalin, β-propiolactone, ethylene oxide, free chlorine, and 70% alcohol all inactivate rubella virus (50, 51).

EPIDEMIOLOGY

Geographic Distribution

Rubella has a worldwide distribution, although clinically recognized disease occurs less frequently in tropical regions than in temperate zones (52). Because humans are the only known natural host for rubella virus, the virus must circulate continuously within populations of people between periods of epidemics. Such endemic spread of rubella virus occurs in most areas of the globe, although small islands that are geographically isolated can lack endemnicity (1, 53). Rubella epidemics have occurred as well on several large islands without the establishment of subsequent endemic spread of the virus among the island populations (41, 54, 55).

Incidence and Prevalence of Infection

Reporting of rubella was not required in the United States until 1966. Since widespread vaccination programs were initiated only 3 years later, there is a paucity of thorough U.S. data on the incidence and prevalence of rubella in the prevaccination era. The majority of cases of rubella prior to 1969 occurred in children 5 to 9 years of age (56). In comparison, rubella outbreaks in recent years predominantly have occurred in adolescents and young adults, with 49 to 63% of cases in 1992 and 1993 occurring in persons of at least 20 years of age (57).

Before 1969, epidemics of rubella occurred in 6- to 9-year intervals, with worldwide pandemics ensuing every 10 to 30 years (47). An individual epidemic usually lasted 3 to 4 years, with cases peaking at the middle of the cycle (58). The widespread use of rubella vaccine has interrupted this epidemic pattern in those countries with effective vaccination

programs. The last epidemic of rubella in the United States occurred in 1964 as part of the worldwide pandemic of 1962 to 1965. During that epidemic, 12.5 million cases of rubella were reported in the United States (59), with some 20,000 cases of congenital rubella syndrome (41).

Rubella is a highly contagious disease, and the incidence of rubella infection during an epidemic cycle approaches 100% of susceptible hosts in closed populations (e.g., military recruits) (60–62). Virtually all susceptible household contacts are infected during such outbreaks (54). The overall incidence of disease among susceptible hosts at the community level during an epidemic ranges from 50 to 90% (1). Clinically apparent rubella occurs with equal frequency in boys and girls; however, rubella is more commonly diagnosed in women than men in adult populations, possibly due to heightened awareness of the risk of congenital rubella among women of childbearing age (46).

At least half of all serologically confirmed childhood primary rubella infections result in clinically inapparent illness (41, 61). Reinfection with rubella virus can occur following natural infection, but is usually asymptomatic (62). Viremia is rarely documented, although systemic symptoms such as arthritis and rash may occur (63). Reinfection following vaccination occurs more commonly than following natural infection, but also usually is asymptomatic and not associated with viremia (64, 65). Up to 80% of persons previously vaccinated against rubella will be reinfected during an epidemic (62, 64). Reinfections are more likely to occur in persons with lower rubella antibody titers (62, 64, 65). Rubella reinfection during pregnancy can result in congenital rubella syndrome, although this is a very rare event (66, 67).

The success of the rubella control program is illustrated in the 40 years following licensure of the rubella vaccine in the United States (Figure 1). By 2005, endemic transmission of rubella had been eliminated in the United States (31), and cases of rubella and of congenital rubella syndrome in the United States in 2007 were 99.9 and 99.3% lower, respectively, than in the prevaccine era (68). From 2004 through 2012, 79 cases of rubella and 6 cases of congenital rubella syndrome, including 3 cases in 2012, were reported in the United States; all of the cases were import-associated or from unknown sources (69). An estimated 95% of the U.S. children and adolescents of 6 through 19 years of age are immune to rubella (70). However, approximately 10% of adults of 20 through 49 years of age lack antibodies to rubella, although 92% of women are seropositive.

The remarkable success in decreasing the incidence of rubella in this country and others has led scientists and international organizations to consider the goal of rubella eradication (71–75). In 2003, the Pan American Health Organization (PAHO) adopted a resolution calling for elimination of rubella and CRS in the Americas by the year 2010. All countries with endemic rubella in the Americas implemented the recommended PAHO strategy by the end of 2008. The strategy consists of achieving high levels of measles-rubella vaccination coverage in the routine immunization program and in the supplemental vaccination campaigns to rapidly reduce the number of people in the country susceptible to acute infection. This is accomplished while simultaneously strengthening epidemiologic surveillance to monitor impact. The last confirmed endemic case in the Americas was diagnosed in Argentina in February 2009. In September 2010, the PAHO announced that the region of the Americas had achieved the rubella and CRS elimination goals on the basis of surveillance data, and in 2015, the Americas region became the first WHO region in the world to be declared free of endemic transmission of rubella (32). Rubella continues to circulate in other parts of the world, however, including a large outbreak in Japan occurring since 2012 (76). Approximately 100,000 cases of congenital rubella syndrome still occur worldwide, illustrating the length remaining toward the ultimate goal of a world free of rubella's devastating effects during pregnancy (77). Three of the five remaining WHO regions have set control or elimination targets for rubella (77). The European region has a target of rubella elimination by 2015, and the Western Pacific region aims to have substantially accelerated rubella control and congenital rubella syndrome prevention by 2015. Rubella control or elimination goals are yet to be established in the African, Eastern Mediterranean, and South-East Asia regions.

Seasonality

In temperate climates such as North America and Europe, rubella is most prevalent in March, April, and May (78). This seasonal pattern occurs both in years with high rates of infection and in years with low rates of infection (1).

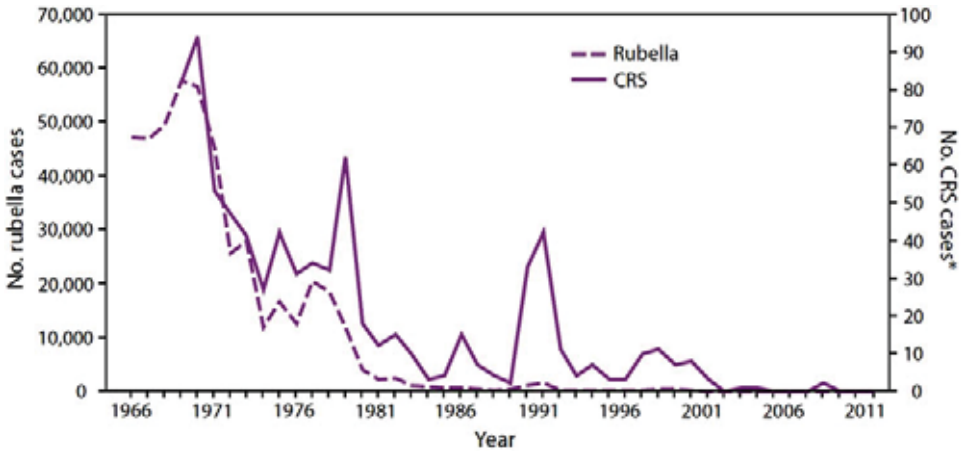

FIGURE 1 Number of rubella and congenital rubella syndrome (CRS) cases—United States, 1966–2011. Source: Rubella and CRS data provided were reported voluntarily to CDC from state health departments. * By year of birth. (Reprinted from reference 162 with permission.)

Transmission

Rubella virus is transmitted primarily by virus-laden droplets from the respiratory secretions of infected persons. In studies conducted with volunteers, rubella virus can be detected in nasopharyngeal secretions from 7 days prior to 14 days following the onset of the rash (79, 80), with maximal shedding of virus occurring from 5 days before to 6 days after the appearance of the exanthem (1). The incubation period of rubella is usually 16 to 18 days, but can range from 14 to 23 days (81). Persons with subclinical cases of rubella are contagious and can transmit infection to others. Recipients of the rubella vaccine do not transmit rubella, however, even though the virus can be isolated from the pharynx.

Infants with congenital rubella syndrome are capable of transmission of virus to susceptible persons (82). At one year of age, between 10 and 20% of infants with congenital rubella syndrome continue to shed virus in the nasopharynx (83). Viral shedding occurs for as long as 20 months after birth in up to 3% of congenitally infected infants (84), a finding that can be of particular concern in hospital environments (85).

Individuals vary in their ability to transmit rubella. A minority of patients who have a high likelihood of transmitting virus to susceptible contacts have been identified during rubella epidemics ("spreaders") (86). In contrast, most individuals transmit rubella virus less efficiently ("nonspreaders"). Genetic factors may correlate with the ability to transmit rubella virus, with persons bearing the major histocompatibility complex antigens HLA-A1 and HLA-A8 being more likely to spread rubella during infection (1, 87).

Congenital Rubella

Fetal infection can occur throughout pregnancy, with the risk of infection being greatest during the first trimester, decreasing during the second trimester, and then rising again as the fetus approaches term. In one study, the risk of fetal infection in infants whose mothers had rubella during the first trimester was determined to be 81% (Figure 2) (88). The infection rate following second trimester exposure was 39%, and the infection rate was 53% after exposure during the third trimester (88). In a second study, the risk of fetal infection following maternal rubella during the second trimester was 32%; the risk following third trimester maternal infection was 24% overall, but when maternal infection occurred near term, that rate rose to 58% (89).

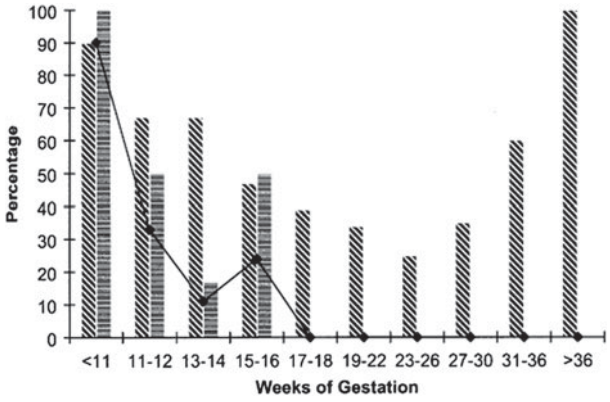

FIGURE 2 Likelihood and outcome of congenital rubella syndrome as a function of time of acquisition of maternal rubella virus infection. Hatched bars, rate of fetal infection; striped bars, rate of defects in infected persons; diamonds, overall risk of defects after maternal infection. (Adapted from reference 88 with permission.)

The risk of congenital anomalies in live-born children following fetal infection also varies according to the month of pregnancy in which maternal infection occurs. One study reported that 85% of infants born to women infected with rubella virus during the first 8 weeks of pregnancy had anomalies detected during the first 4 years of life (90). Detectable defects occurred in 52% of infants born to mothers infected at 9 to 12 weeks' gestation, in 16% of infants born to women infected at 13 to 20 weeks' gestation, and in no infants born to mothers infected beyond 20 weeks' gestation (90). In another investigation that followed infected infants until 2 years of age, 100% (9 of 9) of infants infected within the first 11 weeks gestation had detectable congenital defects (88). In addition, 50% (2 of 4) of infants infected from 11 to 12 weeks' gestation demonstrated congenital anomalies; thus, 85% (11 of 13) infants infected during the first trimester had detectable defects in this study (Figure 2) (88). A study of congenital rubella syndrome among children born to Amish women during the rubella outbreak of 1990 to 1991 reported a similar rate of defects (80%) among congenitally infected infants whose mothers had first trimester infections (91).

For counseling purposes, determination of the risk of congenital defects after confirmed maternal infection can be calculated by multiplying the rates of fetal infections by the rates of defects in infected infants. Accordingly, the risks are 90% for maternal infection before the 11th week of gestation, 33% for infection occurring during weeks 11 and 12, 11% for infection from weeks 13 to 14, and 24% for infection between weeks 15 and 16 (47).

PATHOGENESIS IN HUMANS

Virus Replication

Following initial infection of cells of the nasopharyngeal respiratory epithelium, rubella virus spreads rapidly to the regional lymph nodes by means of the lymphatics and possibly by transient viremia. Viral replication continues in localized areas of the nasopharynx and regional lymph nodes for another 7 to 9 days, followed by viremic spread to multiple sites throughout the body (47). Maximal viremia and viruria occur 10 to 17 days after infection, and heavy viral shedding from the nasopharynx continues from 10 to 24 days postexposure (1).

Rash develops 16 to 18 days after infection. At the same time, antibody begins to be detected, in association with clearance of viremia (47). Virus in tissues also clears rapidly as antibody becomes detectable. While virus can usually be cultured from nasopharyngeal secretions from 7 days before to 14 days after the onset of the rash, maximal viral transmission occurs during the period from 5 days prior to 6 days after the appearance of the rash (47). Other sites from which rubella virus has been cultured include lymph nodes, urine, cerebrospinal fluid (CSF), the conjunctival sac, breast milk, synovial fluid, lung tissue, and skin (at sites both with and without rash) (1).

Histopathology

Postnatal Rubella

Rubella acquired in the postnatal period typically is a mild disease, and death as a consequence of postnatally acquired

rubella is exceedingly uncommon. From 1966 to 1975, 0.05% of reported cases of rubella in the United States resulted in death (92). As a result, a paucity of information exists on the tissue pathology that results from postnatally acquired rubella. Reported morphological changes in lymphoreticular tissues, central nervous system specimens, and synovial tissue have been nonspecific (1). Follicular hyperplasia and edema in lymph nodes and splenic tissue have been documented. In addition, minimal meningeal and perivascular exudate has been noted in neural tissue from a fatal case, as has diffuse swelling and nonspecific degeneration of brain (1). Lymphocytic infiltration, increased vascularity, and synovial cell hyperplasia are seen on synovial biopsy specimens from patients with rubella arthritis.

Congenital Rubella

While direct cellular destruction by rubella virus accounts for some of the tissue damage seen in congenital rubella syndrome, vascular injury and resulting insufficiency are more important in the pathogenesis of congenital defects (47, 93, 94). The amount of inflammation produced in target organs is much less than that seen with other congenital viral infections. In addition, rubella virus infection *in vitro* disrupts actin microfilaments (95), and mitotic arrest has been demonstrated *in vivo* (96). Such disruption and arrest may account for the decreased numbers of cells in many organs of congenitally infected infants, resulting in their generalized intrauterine growth restriction.

The pathological findings of the placenta include extensive perivasculitis, endovasculitis, and perivascular fibrosis (93, 94). Edema, fibrosis, and necrosis of the chorionic villi also occur, resulting in a small placenta. Cellular necrosis and other evidence of cytolysis also are present, but are less widespread than the vascular lesions.

Numerous organs are involved in congenital rubella syndrome. In general, affected organs are hypoplastic, in part due to the reduction in the total numbers of cells. The necrotizing angiopathy of small blood vessels is characteristically seen in affected organs, including the placenta. Cellular and tissue necrosis also can be demonstrated in affected organs, although much less frequently than the vascular findings. As would be expected with a chronic infection, new and old lesions frequently can be seen in a single tissue specimen (93, 94).

Immune Responses to Rubella Virus Infection

Postnatal Rubella

Humoral Immune Response. Rubella-specific immunoglobulin M (IgM) antibodies can be detected by hemagglutination inhibition assay (HAI), immunofluorescence assay (IFA), radioimmunoassay, or enzyme-linked immunosorbent assay (ELISA) (47, 97). IgM antibodies can usually be detected within a few days of the onset of the rubella rash (Figure 3). After a rapid peak, however, the IgM component of the host antibody response rapidly declines, becoming undetectable by 8 weeks following initial infection. Rarely, patients can have persistence of rubella-specific IgM for prolonged periods (98). IgM is usually not seen with reinfection. When IgM is present, reinfection can be distinguished from primary rubella by testing of the avidity of the IgG produced, which is higher in reinfection (99, 100).

Neutralizing and HAI IgG antibodies are first detectable in serum 14 to 18 days following infection, at the time of the rash (79). The quantity of HAI antibodies peaks around 2 weeks later and then gradually declines over the following

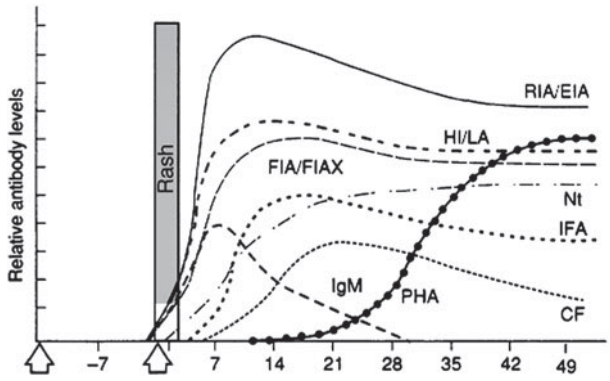

FIGURE 3 Schematic of the immune response in acute rubella virus infection. Values on the *x* axis indicate number of days. RIA, radioimmunoassay; EIA, enzyme immunoassay; HI, hemagglutination inhibition; LA, latex agglutination; FIA/FIAX and IFA, immunofluorescence; Nt, neutralization; PHA, passive agglutination. (Reprinted from reference 47 with permission.)

year and persists thereafter for life. IgG antibodies measured by latex agglutination, neutralization, IFA, single radial hemolysis, RIA, and ELISA generally parallel this HAI pattern of IgG antibody kinetics (Figure 3) (47). Passive hemagglutination IgG antibodies become detectable somewhat later, at 3 to 4 weeks following onset of rash (1). These antibodies then slowly rise to peak levels over the ensuing weeks, and probably persist for life (47). Complement fixation (CF) IgG antibodies are first detectable 7 to 10 days after the onset of the rash, peaking at 1 to 3 months. CF antibodies subsequently diminish to the point of being undetectable over several years in the majority of patients.

The principle IgG subclass detected by the above assays is IgG1 (101). In addition, IgA mucosal HAI and neutralizing antibodies are usually produced following postnatally acquired rubella infection. Roughly half of patients who receive the rubella vaccine RA27/3 subcutaneously will produce detectable amounts of rubella-specific nasal IgA antibody (1).

Cellular Immune Response. Cell-mediated immune responses following postnatal rubella infection can be detected by lymphocyte transformation response, secretion of interferon or macrophage migration inhibitory factor, induction of delayed hypersensitivity to skin testing, and release of lymphokines by cultured lymphocytes (47). Cell-mediated responses can usually be demonstrated 1 week prior to the initiation of humoral immunity, peaking about 2 weeks after the onset of rash and then persisting for years (47). Transient suppression of lymphocyte function can occur initially, thus explaining the suppressed response to purified protein derivative within the month following acute rubella virus infection (102).

Congenital Rubella

Humoral Immune Response. Following maternal rubella infection, the transplacental transfer of maternal IgG is minimal during the first half of pregnancy but increases considerably beginning around 16 to 20 weeks' gestation. As a consequence, until the middle of the second trimester, the amount of maternal rubella-specific IgG present in the fetal circulation is only 5 to 10% of that present in the maternal circulation (47). At roughly the same time that transplacental transport of rubella-specific IgG is increasing at

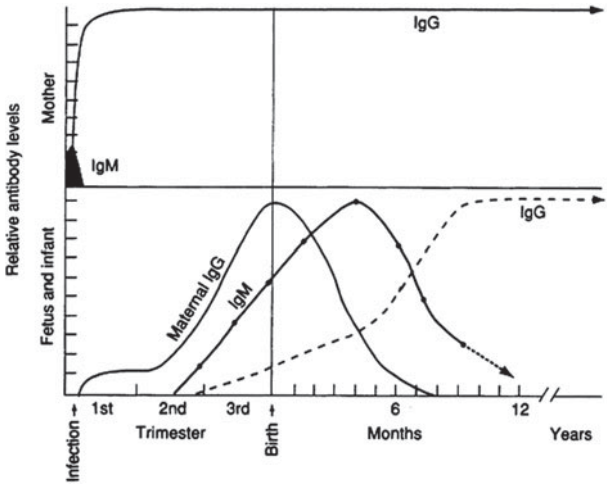

FIGURE 4 Schematic of the immune response in the mother, fetus, and infant after maternal and fetal rubella virus infections in the first trimester of pregnancy. (Reprinted from reference 47 with permission.)

midgestation, the fetal humoral system is beginning to produce detectable quantities of fetal immunoglobulin. The predominant class of fetal antibody produced in the latter half of pregnancy is IgM, although fetal IgG and IgA are also made (Figure 4) (103). Nevertheless, rubella-specific IgG is more abundant overall, due to the combined amounts of both maternal and fetal antibody of this class. As the concentrations of maternal IgG decline following birth, rubella-specific IgM will predominate for a period of several months before declining to levels that are less than those of the increasing neonatal IgG. Virtually all congenitally infected infants have detectable IgM during the first 3 months of life; IgM is detectable in about half of such infants between 3 and 6 months of age; and about one-third have detectable IgM from 6 months to 2 years of age (104). Over the first several years of life, the amounts of rubella-specific IgG can decrease markedly, and some children can lose detectable amounts of such IgG altogether (105, 106). Low avidity IgG can persist even after the disappearance of rubella-specific IgM (107).

Cellular Immune Response. Rubella-specific cell-mediated immune responses (lymphocyte-mediated cytotoxicity, lymphocyte transformation, lymphocyte interferon production, and leukocyte migration inhibition factor production) in infants with congenital rubella are diminished compared to those of children following postnatally acquired disease (1, 108). Additionally, abnormal delayed-hypersensitivity skin reactions can occur in congenitally infected persons (47). The degree of cellular immune dysfunction is greater in children exposed to rubella virus earlier in gestation, with the greatest impairment noted in infants whose mothers were infected during the first 8 weeks of pregnancy (108).

CLINICAL MANIFESTATIONS

Postnatal Rubella

In children who acquire rubella postnatally, a distinct prodromal period is rare. Adolescents and adults, on the other hand, usually will have prodromal symptoms from 1 to 5 days prior to the development of the rash. Symptoms include some combination of lymphadenopathy, low-grade fever, ophthalmalgia, mild conjunctivitis, headache, malaise, anorexia, aches, chills, cough, coryza, and sore throat. Lymph nodes that are characteristically involved include the suboccipital, posterior auricular, and posterior cervical chains. Such nodes are usually painful in adults. In volunteer studies with young adults, it was found that the lymph node enlargement usually lasts from 5 to 8 days (109), although resolution may take several weeks. While frequently occurring together in older patients with rubella, the combination of suboccipital lymphadenopathy and rash is not pathognomonic for rubella virus infection.

Up to half of all serologically confirmed childhood primary rubella virus infections result in subclinical illness (41, 61). In those patients who do develop symptoms, the rash usually appears after an incubation period of 16 to 18 days. The exanthem initially appears on the face and then spreads rapidly to the trunk and distal extremities. The erythematous, maculopapular rash usually does not coalesce, and it typically spreads to the entire body within 24 h. During the second day of the exanthem, the rash begins to fade on the face, and by the end of the third day, it has resolved across the entire body (hence the term "*three-day measles*"). The rash frequently is pruritic, especially in adults. Desquamation can occur during the convalescent stage of disease. If the patient was febrile during the prodromal period, defervescence usually occurs within 1 day of the appearance of the rash.

Roughly 100 years ago, Forcheimer (110) described an enanthem of rubella consisting of small "rose red spots" on the soft palate and uvula that fade within 24 h, "sometimes leaving a yellowish brown pigmentation." In another article, however, Forcheimer refers to the enanthem as "small, discrete, dark red, but not dusky papules which disappear in a short time, leaving no trace behind" (111). Regardless of the type of enanthem noted, neither is pathognomonic for rubella, in contrast to the Koplik spots of measles.

Patients with postnatally acquired rubella frequently have leukopenia at the time of onset of rash. Studies in adult volunteers documented leukopenia beginning 1 day before the onset of rash and persisting for 4 to 5 days (109). Elevation of the erythrocyte sedimentation rate can develop during the first week of illness (1).

Recently, rubella virus has been associated with Fuchs heterochromic iridocyclitis (112, 113).

Complications

Arthritis and Arthralgias. Joint manifestations occur commonly following natural rubella virus infections in adults. Acute polyarticular arthritis occurs in 33 to 52% of women with natural rubella disease, while only 9 to 10% of men experience acute arthritis following rubella infection (114). Symptoms range from joint pain alone to frank arthritis, with swelling, joint effusions, decreased articular mobility, and local warmth and erythema. Joint complaints begin 1 to 6 days after the onset of the rash and can take several weeks to resolve completely (1). Chronic arthritis can develop, although this occurs infrequently. Arthritis and arthralgias can also occur following rubella immunization, although at a lower frequency than following infection with wild-type virus (114).

The pathogenesis of rubella arthritis is not fully understood. Some studies have implicated circulating immune complexes in disease pathogenesis (115), while others have found no such direct role (116). Rubella virus has been cultured from the synovial fluid of patients with acute and recurrent rubella arthropathies (117, 118), as well as from peripheral blood mononuclear cells of patients with chronic

arthritis (117). One patient with chronic polyarthritis had persistent synovial lymphocyte proliferative responses to rubella antigen for at least 7 years following disease onset, raising the possibility of ongoing rubella antigen production within the joint (118, 119). Persistent rubella virus infection has been achieved in cultured human joint tissue, suggesting the possibility that similar events may occur *in vivo* (120, 121).

Neurologic Involvement. Unlike the arthropathies described previously, encephalitis and postinfectious encephalopathy are very rare complications of natural postnatal rubella virus infection. The rate of occurrence of such neurologic events ranges from 1 in 4,700 to 1 in 6,000 cases of rubella (122, 123). Neurologic symptoms appear abruptly 1 to 6 days after appearance of the rash, with headache, vomiting, lethargy, nuchal rigidity, and generalized seizures. CSF white blood cell counts range from 20 to 100 cells/mm^3, with a lymphocyte predominance; CSF protein concentrations are normal or slightly increased; and CSF glucose concentrations are normal (1). Electroencephalographic tracings are frequently abnormal (41).

Mortality rates due to the neurologic manifestations of rubella range from 20 to 50% (124). Survivors usually recover fully following disease resolution (41). The pathogenesis of such complications is unclear, with some reports suggesting a direct involvement of rubella virus and others reporting findings consistent with a postinfectious process.

Hemorrhagic Manifestations. While transient depression of the thrombocyte count occurs not infrequently in postnatal rubella, thrombocytopenic purpura is encountered in only 1 in 1,500 cases (122). The median interval between onset of the exanthem of rubella and development of purpura is approximately 4 days (1). Unlike the other complications of rubella discussed previously, hemorrhagic manifestations of disease are more likely to occur in children than adults. The thrombocytopenia can last for weeks or months, but complete recovery eventually results in most cases.

Differential Diagnosis

The benign nature of rubella virus infection and its nonspecific symptoms contribute to the difficulty in diagnosing rubella on clinical grounds. In addition, the marked decrease in disease incidence has resulted in many physicians lacking personal experience in recognizing the disease. Consequently, rubella can be readily mistaken for such illnesses as scarlet fever, toxoplasmosis, infectious mononucleosis, measles, roseola, erythema infectiosum, and enteroviral infections (124). In adults, the pruritic component of the rubella exanthem can be confused with an allergic reaction.

Congenital Rubella

Unlike those of postnatal rubella disease, the clinical manifestations of congenitally acquired rubella usually are severe. The classic triad of congenital rubella consists of cataracts, cardiac abnormalities, and deafness. In addition, less frequent manifestations of congenital rubella syndrome were recognized during the large pandemic of 1962 to 1965 and are collectively referred to as the expanded congenital rubella syndrome, as detailed below.

The consequences of *in utero* rubella virus infection can be considered broadly as belonging to one of three categories: (i) signs and symptoms that are transiently apparent in affected infants, (ii) permanent manifestations that are noted within the first year of life, and (iii) manifestations of congenital rubella that are delayed in onset until later in life (2 years of age to adulthood) (47).

Transient Sequelae

Many of the transient clinical manifestations of congenital rubella were first recognized during the large pandemic of 1962 to 1965. As implied, these manifestations usually resolve over a period of weeks. They include dermal erythropoiesis ("blueberry muffin" rash), chronic rash, thrombocytopenic purpura, hemolytic anemia, generalized lymphadenopathy, interstitial pneumonitis, hepatitis, hepatosplenomegaly, nephritis, myositis, myocarditis, bone radiolucencies, and meningoencephalitis (1). Among the more common of these findings are rash (petechial or "blueberry muffin" rash), hepatosplenomegaly, jaundice, pulmonary involvement, meningoencephalitis, and radiographic abnormalities (Figure 5) (1). The majority of such infants are intrauterine growth restricted at delivery (47).

Permanent Manifestations

Sensorineural hearing loss is the most common permanent manifestation of congenital rubella, with deafness occurring in 80% of congenitally infected patients (47). Additional permanent sequelae of congenital rubella include cardiovascular anomalies, ophthalmologic findings, and neurologic impairment.

Structural defects of the cardiovascular system occur in the majority of infants whose mothers acquired rubella during the first 2 months of gestation (1). Patent ductus arteriosus is the most common of these cardiovascular sequelae, followed by pulmonary artery stenosis, and pulmonary valvular stenosis. Two-thirds of patients with patent ductus arteriosus will have other cardiovascular lesions present (1).

Ophthalmologic findings include cataracts (bilateral or unilateral), retinopathy, and microphthalmia. The retinopathy results from pigmentary defects in the retina and usually does not interfere with vision. In contrast, a small number of patients have congenital glaucoma that, if undetected, can result in visual impairment.

Permanent neurologic impairment can result from the active replication in the central nervous system of rubella virus both *in utero* and following delivery. Indeed, such neurologic sequelae as mental retardation and motor disabilities correlate with the severity and persistence of the

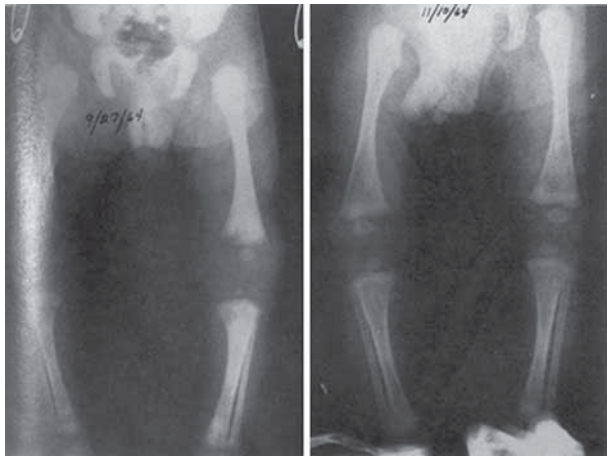

FIGURE 5 (Left) Provisional zones of calcification are poorly defined and irregular. Radiolucent defects are present in metaphyses of femora and tibiae and the parallel long axis of the bone. (Right) Lower extremities 2 months later show nearly complete disappearance of osseous abnormalities. (Reprinted from reference 163 with permission.)

acute meningoencephalitis that is present at delivery in 10 to 20% of infants with congenital rubella syndrome (1). Movement and behavioral disorders can also be seen in surviving patients.

Delayed Manifestations

Sequelae of congenital rubella that develop in childhood or adulthood but are not present in infancy include endocrinopathies, deafness, ocular damage, vascular effects, and progressive rubella panencephalitis (125, 126). Of these, the development of insulin-dependent diabetes mellitus occurs most frequently, with approximately 20% of patients being diagnosed with this form of diabetes by the time they reach adulthood (47). Autoimmune-mediated thyroid dysfunction can also be seen (127).

LABORATORY DIAGNOSIS

Rubella virus infection is definitively diagnosed by isolation of rubella virus in tissue culture, using one of several cell lines and primary cell strains. Viral interference in AGMK cells is one common culture technique by which the presence of rubella virus is demonstrated. When using such interference techniques, the presence of rubella virus is then confirmed by the specific detection of viral antigen by neutralization or immunofluorescence.

Virus can be readily isolated from throat swabs from patients with postnatal rubella virus infection for 6 days before and after the onset of rash (41, 64). Virus can be isolated from specimens from the nasopharynx, conjunctivae, urine, blood buffy coat, and CSF of patients with congenital rubella. *In utero* infection with rubella virus can be demonstrated by nucleic acid hybridization or by virus-specific antigen detection in specimens from the chorionic villus or fetus (128, 129). PCR assays have also been developed, and detection of rubella virus RNA by real time reverse-transcriptase PCR from a throat/nasal swab or urine sample with subsequent genotyping of strains may be valuable for diagnosis and molecular epidemiology (130).

Despite the definitive results afforded by direct viral isolation in tissue culture, the majority of rubella cases are diagnosed serologically. A 4-fold rise in rubella-specific IgG between acute- and convalescent-phase serum specimens confirms the diagnosis of postnatal rubella. Commercially available rubella virus IgG avidity assays are of variable sensitivity (131). Demonstration of the presence of rubella-specific IgM is also diagnostic for recent infection with rubella virus. Rarely, rubella-specific IgM can be detected with reinfection. Specific antigens of rubella virus can be identified by such serologic reactions as CF (132), hemagglutination inhibition assay (HIA) (133), precipitation (134), platelet aggregation (135), IFA (136), and ELISAs (137). Although the HAI remains the reference standard by which other tests are compared, simpler tests such as ELISAs have become the predominant assays used by commercial laboratories for the detection of rubella-specific IgG or IgM.

Serologic diagnosis of congenital rubella can be demonstrated by the presence of rubella-specific IgM in neonatal serum. Confirmation of the diagnosis based solely upon the presence of IgG is difficult. In such cases, it is necessary to test sequential sera from the infant for rubella-specific IgG. In most cases, the IgG titer will decrease over several months if it is solely of maternal origin, whereas it will rise if congenital infection has occurred and the infant is producing rubella-specific IgG.

PREVENTION

Hospitalized patients with postnatal rubella require contact isolation for 7 days after the onset of the rash. Postnatally infected children should be excluded from school or child care for the same period (81). In contrast, infants with congenital rubella should be considered contagious until at least their first birthday, unless two nasopharyngeal or urine viral cultures obtained after 3 months of age and obtained 1 month apart are negative.

Exposure to rubella virus during pregnancy can be especially anguishing (138). If a woman with such an exposure is known to be rubella-immune from a previous pregnancy, she can be reassured with no further evaluation required. If, on the other hand, she is not immune to rubella or her rubella status is unknown, serologic testing should be performed immediately. If such testing performed around the time of the exposure demonstrates the presence of rubella antibody, it can be assumed that she is immune and thus not at risk. However, if no rubella-specific antibody is detected, she should have a second serum sample obtained 2 to 3 weeks after the exposure, and it should be tested for antibody simultaneously with the first specimen; seroconversion suggests that infection occurred with the exposure. If the second test is also negative, a final serologic analysis should be performed on a serum sample obtained 6 weeks following the initial exposure and also tested concurrently with the first specimen; a negative test result for both specimens indicates that infection has not occurred, and a positive result for the second but not the first (seroconversion) indicates recent infection (81).

Passive Immunoprophylaxis

Administration of immunoglobulin to susceptible persons experimentally exposed to rubella virus can prevent clinical rubella (139). However, there have also been many reports of the failure of immunoglobulin to prevent the anomalies of congenital rubella (140, 141). Therefore, the routine use of immunoglobulin for the prevention of rubella in an exposed pregnant patient is not recommended (81); administration of immunoglobulin should be considered only if termination of the pregnancy is not an option. Limited data indicate that intramuscular immunoglobulin in a dose of 0.55 ml/kg of body weight may decrease clinically apparent infection in an exposed susceptible person from 87 to 18% compared with placebo. However, the absence of clinical signs in a woman who has received intramuscular immunoglobulin does not guarantee that fetal infection has been prevented (81).

Active Immunization

Since 1979, the RA27/3 rubella vaccine has been used exclusively in the United States (29, 30). Vaccination with a single dose of vaccine at 12 months of age or older results in IgG antibody production in more than 95% of vaccine recipients, and a single dose confers long-term (probably lifelong) immunity against clinical and asymptomatic infection in more than 90% of vaccines (81). Because of the two-dose recommendations for measles- and mumps-containing vaccine (as MMR) and varicella vaccine (as MMRV), two doses of rubella vaccine are administered routinely. This provides an added safeguard against primary vaccine failures. Cellular and humoral immune responses to rubella vaccination may be influenced by the HLA alleles of the vaccine recipient (142, 143). Subcutaneous administration of vaccine induces production of IgM antibodies that peak at 1 month post-vaccination (104).

Rubella vaccine given after exposure to wild-type rubella virus theoretically can prevent illness if administered within 3 days of exposure (81). Immunization of exposed nonpregnant persons may be indicated because, if the exposure did not result in infection, immunization will protect the person in the future.

Rubella vaccine is administered subcutaneously in combination with measles and mumps vaccine (MMR) or in combination with measles, mumps, and varicella vaccine (MMRV). Monovalent rubella vaccine and combined rubella-measles vaccine are no longer available in the United States. Current recommendations for rubella vaccination call for administration of the first MMR or MMRV vaccination at 12 to 15 months of age. Antibody responses following the first dose of a rubella-containing vaccine are similar for premature and term infants at 15 months of age (144). A second MMR or MMRV vaccination is then recommended at school entry at 4 to 6 years of age; those persons who have not received this dose at school entry should receive their second dose of a rubella containing vaccine as soon as possible, but no later than 11 to 12 years of age.

Postpubertal females who are not known to be immune to rubella should be immunized. They should not receive the vaccine if they are pregnant, and they should be warned not to get pregnant within 28 days of vaccination. In addition, premarital serologic screening for rubella immunity will bolster attempts at identifying susceptible women of childbearing age. Finally, prenatal or antepartum serologic screening for rubella immunity should be routinely performed. Women who are found to be rubella susceptible should receive rubella vaccine in the immediate postpartum period prior to discharge. Breast feeding is not a contraindication to such immunization. Vaccinated women of childbearing age who have received one or two doses of rubella-containing vaccine and have rubella serum IgG concentrations that are not clearly positive should be administered one additional dose of MMR vaccine (maximum of three doses) and do not need to be retested thereafter for serologic evidence of rubella immunity (81).

Adverse Reactions

From 5 to 15% of children receiving rubella vaccine develop rash, fever, or lymphadenopathy between 5 and 12 days after vaccination. Approximately 0.5% of children and 25% of susceptible postpubertal female vaccinees develop arthralgias beginning 7 to 21 days after vaccination. Such symptoms usually involve small peripheral joints. The incidence of joint manifestations after vaccination is lower than that following natural infection at the corresponding age (81). MMR vaccination rarely can cause idiopathic thrombocytopenic purpura, with cases occurring in approximately 1 in 22,000 doses; children with a history of idiopathic thrombocytopenic purpura do not have relapses following receipt of MMR (145).

A possible relationship between rubella vaccination and chronic arthritis in adult women is controversial. Following a 20-month review, the Institute of Medicine (IOM) in 1991 found that the evidence is consistent with a causal relation between the RA27/3 rubella vaccine strain and chronic arthritis in adult women, although the evidence is limited in scope (146). In a subsequent special report, the IOM stated that "proving that rubella vaccination can cause chronic arthritis will require an understanding of pathogenetic mechanisms and additional well-designed studies" (147). One such large retrospective cohort study was published in 1997 and found no evidence of any increased risk of new onset chronic arthropathies or neurologic conditions in women receiving the RA27/3 rubella vaccine (148). Other reports also have not demonstrated a definitive association between rubella vaccine and persistent or recurrent joint manifestations, although additional investigations are needed to definitively disprove such a relationship (149, 150).

In 1998, a possible link between MMR vaccine and inflammatory bowel disease was published by Wakefield et al. (151). Over the ensuing years, their results could not be reproduced by others, and in 2004 it came to light that Wakefield's work had actually been funded by lawyers in Britain who were filing class action lawsuits regarding such claims. In 2004, 10 of the 12 coauthors of the original paper published a retraction (152), and the editors of *The Lancet* detailed these misgivings and others in an editorial (153).

Another allegation of the initial Wakefield paper from 1998 was that MMR was also associated with autism. Again, numerous studies subsequently found no such association, leading to an Immunization Safety Review Committee of the IOM review of the epidemiologic and other evidence on MMR vaccine and risk for autism spectrum disorders. The conclusion of the IOM was that the evidence favors rejection of a causal relationship (154). Despite the unequivocal conclusions of the scientific community, confusion and doubt remain within the general population (155), illustrating the dangers of recklessly raising safety concerns for vaccines that have had such a positive impact on human health worldwide.

Precautions and Contraindications

Rubella vaccine should not be administered to pregnant women, although data from CDC surveillance systems have not detected adverse outcomes if vaccine is inadvertently provided during pregnancy (156). Based upon data from the Centers for Disease Control and Prevention, the maximal theoretical risk for the occurrence of congenital rubella syndrome following administration of the RA27/3 vaccine during the first trimester of pregnancy is 1.3% (81). While asymptomatic rubella infection has been reported for 2% of such infants, no cases of congenital rubella syndrome resulting from live-virus vaccination of the mother have been reported (81). Persistence of fetal infection following inadvertent rubella vaccination during early pregnancy has been documented, but with no apparent adverse clinical sequelae (157).

Patients with altered immunity should not receive the rubella vaccine. These include patients with immunodeficiency diseases (except human immunodeficiency virus infection), patients receiving immunosuppressive therapy, and patients receiving large systemic doses of corticosteroids, alkylating agents, antimetabolites, or radiation. If possible, children receiving biologic response modifiers, such as anti-tumor necrosis factor-alpha, should be immunized prior to initiating treatment. Immunocompetent children with minor illnesses with or without fever may be vaccinated. Rubella vaccine should not be given in the 2 weeks prior to or up to 7 months following the administration of immunoglobulin or blood products.

TREATMENT

Postnatal rubella virus infection is usually either subclinical or so mild that no therapy is warranted. Complications of rubella virus infection can be treated symptomatically. Management of rubella arthritis in adults may require bed rest and administration of aspirin or nonsteroidal anti-inflammatory

agents. Likewise, postinfectious encephalopathy and encephalitis are managed with supportive care, as are the thrombocytopenic and hemorrhagic manifestations of rubella.

Patients with congenital rubella require supportive care not only in the neonatal period but throughout life for such permanent impairments as deafness and heart defects. Prompt identification of such afflictions is of the utmost importance.

Interferon and amantadine have been used in individual cases of congenital rubella syndrome (158, 159). Results have been equivocal, however, and no controlled trials have been performed. Interferon has also been used in the treatment of chronic arthritis secondary to postnatal rubella virus infection, again with indeterminate results (160). Isoprinosine has been administered to patients with progressive rubella panencephalitis, without apparent therapeutic benefit (161).

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Infections Caused by Bornaviruses

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Bornaviruses (*Mononegavirales*: *Bornaviridae*) form enveloped virions with nonsegmented, single-stranded negative-sense genomes (~8.9 kilobases). They naturally infect mammals (e.g., bicolored white-toothed shrews [*Crocidura leucodon*], equids, sheep, variegated squirrels [*Sciurus variegatoides*] but rarely other mammals including humans) and a wide variety of birds and snakes. Bornaviruses have unique characteristics, such as 1) replication in the nucleus using cellular splicing machinery for generation of mRNAs and integrating bornaviral elements into the host-cell genome; 2) genome trimming for generation of RNAs that probably do not trigger innate immune responses in infected cells; and 3) suppression of apoptosis in infected cells mediated by the accessory protein (X), leading to persistent noncytolytic infection.

Bornaviruses can infect a wide spectrum of nerve cells and other cells of mammals, birds, reptiles, and most probably animals of other orders. Humans and other mammals bear endogenous bornaviral elements in their genomes as a result of infections of early ancestors with ancient bornaviruses. Aggravated $CD8^+$ T-cell responses to the bornaviral nucleoprotein (N) can result in immunopathological disease in infected animals. Infected newborn animals are immunotolerant and do not develop $CD8^+$ T-cell responses, leading to persistent infection.

The number of known bornaviruses is increasing, but the epidemiology of bornavirus infections remains poorly understood. The detection of a new, divergent bornavirus associated with fatal neurological disease in three patients calls for further study of human bornavirus infections.

VIROLOGY

Classification

Bornaviruses are assigned to the only genus of the mononegaviral family *Bornaviridae*, *Bornavirus* (1). Several bornavirus species have been established (2), but other bornaviruses are still unassigned due to limited information about their genetic and biological properties (Table 1).

Serotypes and Antigenicity

Replicating virus isolates are available for mammalian and bird bornaviruses discovered to date. Reptile bornaviruses are only known from genome detection and sequencing. Current knowledge about bornavirus serotypes is therefore limited to mammalian and avian representatives of the family.

Antibodies directed against N protein, X protein, phosphoprotein (P), and matrix (M) protein cross-react well between avian and mammalian bornaviruses (3). The immune response directed against the glycoprotein (G) is linked to virus-neutralizing activity (4, 5). Defining serotypes for bornaviruses is not yet possible. Although there are differences in the degree of cross-reactivity, currently the existence of only one serotype is supported by measuring immune responses against N, X, P, and M proteins (3). Passeriform and mammalian bornaviruses have more prominent cross-reactivity to each other than to psittaciform bornaviruses (3). Further investigations are needed to postulate serotypes based on antibody responses to the G protein.

Viruses

Viruses known so far are listed in Table 1. Genetic relationships between the recent mammalian bornaviruses detected in Europe and bornaviruses of birds of the Passeriformes and Anseriformes are closer than those observed in the more genetically distant bornaviruses found in parrots of the Psittaciformes (Figure 1). Bornaviruses vary significantly in the first intergenic region between the genes coding for the N and P protein, which contains a short open reading frame upstream of the X gene (uORF) that is lacking in estrildid finch, parrot, and snake bornaviruses (2).

Endogenous Bornavirus-Like Elements

Bornavirus-related sequences have been detected in the genomes of various mammals and other diverse animal species (6–9) but are less frequent in birds in comparison to mammals (10). These sequences were incorporated into genomes most probably by long interspersed nucleotide elements (LINE) through reverse transcription (6). Consequently, fragments of genetic information of older bornaviruses in the evolutionary lineage are still present in animal genomes (6). Most of these endogenous bornavirus-like elements are related to the Borna disease virus (BoDV) N genes and are designated endogenous Borna-like N elements (EBLNs) (6).

The human genome contains four EBLNs, which are located on chromosomes 3, 9, 10, and 17, respectively (6).

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TABLE 1 Overview of the mononegaviral monogeneric family *Bornaviridae*

Species	Viruses (abbreviations)	Hosts	Disease	Impact for humans
Mammalian 1 bornavirus	Borna disease viruses 1 and 2 (BoDV-1/2)	Bicolored white-toothed shrews and spillover into horses, sheep, New World camelids	Borna disease in spillover hosts	Disease never reported; natural infection uncertain; can easily infect human cell lines; disease induced in experimentally infected rhesus monkeys
Psittaciform 1 bornavirus	Parrot bornaviruses 1, 2, 3, 4, 7 (PaBV-1/2/3/4/7)	Parrots	Proventricular dilatation disease	Disease never reported; natural infection uncertain; infection of cell lines established from humans difficult
*Psittaciform 2 bornavirus**	Parrot bornavirus 5 (PaBV-5)	Parrots	Proventricular dilatation disease	Not known
Waterbird 1 bornavirus	Aquatic bird bornaviruses 1 and 2 (ABBV-1/2)	Wild geese, swans, ducks	Proventricular dilatation disease	Not known
Passerinform 1 bornavirus	Canary bornaviruses 1, 2, 3 (CnBV-1/2/3)	Canary birds	Proventricular dilatation disease	Not known
Passeriform 2 bornavirus	Estrildid finch bornavirus 1 (EsBV-1)	Estrildid finches	None	Not known
*Elapid 1 bornavirus**	Loveridge's garter snake virus 1 (LGSV-1)	Snakes	Not known	Not known
Unassigned	Gaboon viper virus 1 (GaVV-1)	Snakes	Not known	Not known
Unassigned	Munia bornavirus 1 (MuBV-1)	Estrildid finches	Not known	Not known
Unassigned	Parrot bornavirus 6 (PaBV-6)	Parrots	Proventricular dilatation disease	Not known
Unassigned	Parrot bornavirus 8 (PaBV-8)	Parrots	Proventricular dilatation disease	Not known
Unassigned	Variegated squirrel bornavirus 1 (VSBV-1)	Variegated squirrels and spillover into humans	Meningoencephalitis	Fatal infection

* These species have recently been accepted by the International Committee on Taxonomy of Viruses (ICTV), but at the time of this writing they have not yet been ratified.

Two of these EBLNs contain an intact open reading frame that induce proteins that could interact with human functional proteins (6, 11, 12). Some EBLNs are capable of inhibiting replication of other bornaviruses *in vitro* (11), suggesting they may serve as intracellular sensors for and antiviral factors against infection with extant bornaviruses (8). Human EBLN-1 does not prevent the replication of the evolutionarily recent BoDV-1 (11). Genomic incorporation of exogenous bornavirus fragments can be detected by polymerase chain reaction (PCR) without a reverse transcription step directly after experimental and natural infection in cell lines and animals (6, 13, 14).

Composition

Bornaviruses are composed of enveloped spherical particles of 90 to 100 nanometers in diameter and contain an electron-dense core of 50 to 60 nanometers. (15, 16). These particles most probably spread from infected cells in the form of infectious ribonucleoproteins (RNPs) (17).

The genome of bornaviruses is composed of a nonsegmented, linear, single-stranded, negative-sense RNA of approximately 8.9 kilobases that is organized into three transcription units (18, 19). The termini are composed of short, complementary, noncoding sequences. Bornavirus genomes are characterized by overlapping ORFs and transcriptional signals (1). The genomes of bornaviruses encode at least six viral proteins (Table 2): N (20–22), X (23, 24), P (25), M (26), G (4), and the RNA-dependent RNA polymerase L (27).

Biology

Replication Strategy

Bornaviruses replicate and are transcribed in the nuclei of infected cells (28). These viruses use alternative splicing of polycistronic primary transcripts and different transcription and termination signals to generate a wide array of mRNAs (29–31). Replication of bornaviruses results in genomic and

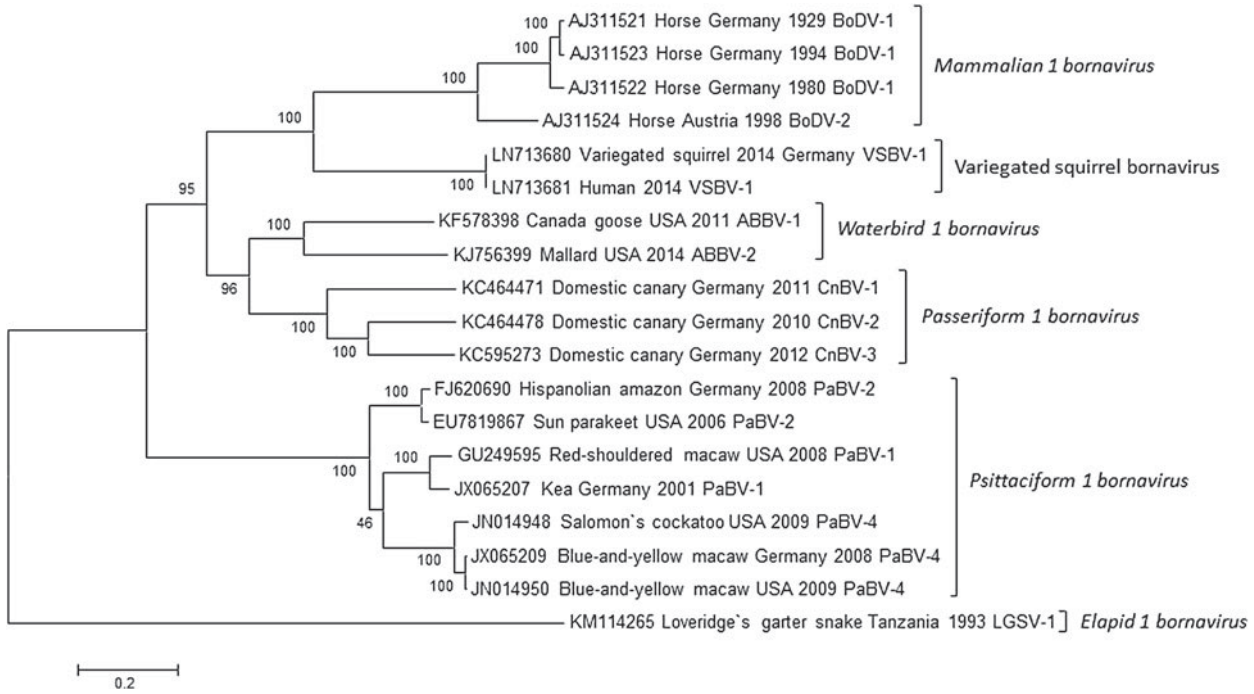

FIGURE 1 Unrooted phylogenetic tree of selected complete bornavirus sequences available at GenBank. Phylogenetic analysis was performed using the MEGA version 5.05 package program. Gene-specific substitution models were evaluated, and best-fit models were selected (Tamura3-parameter model for the tree shown). Maximum-likelihood trees were generated, tree topologies were validated by bootstrap analysis (1000 replicates), and the best phylogenetic tree was selected. The bar represents nucleotide substitutions per site. The taxon information includes the GenBank accession number, the host species, the country of the host, the year of detection, and the virus abbreviation. Furthermore, the virus species are shown according to the International Committee on Taxonomy of Viruses (ICTV) nomenclature, including the newest taxonomic proposals. Variegated squirrel bornavirus has not yet been classified (tentatively *Mammalian 2 bornavirus*).

antigenomic viral RNAs with trimmed 5′ ends that contain monophosphorylated terminal nucleotides (32, 33).

Growth in Cell Culture

Bornaviruses do not cause cytopathic effects (34–37), possibly because the X protein inhibits apoptosis (38).

Inactivation by Physical and Chemical Agents

Like all mononegaviruses, bornavirions are sensitive to organic solvents, detergents, exposure to ultraviolet light, a pH value below 4, and temperatures of 56°C or higher (37, 39–42).

EPIDEMIOLOGY

Host Range, Distribution, and Geography

Bornaviruses are most probably distributed worldwide (43). EBLNs in primates indicate that bornaviruses have existed in Africa for at least 40 million years (6). Bornaviruses are still present in Africa as revealed by the recent detection of a bornavirus in an African snake (44). EBLNs found in thirteen-lined ground squirrels (*Ictidomys tridecemlineatus*) are approximately 6 million years old and hint of an endemic long-term coexistence of bornaviruses with hosts in the Americas. They are genetically more closely related to the N gene of parrot bornaviruses than to that of other known bornaviruses (6, 45). The wide distribution of bornaviruses in waterbirds from different continents (46) indicates aquatic reservoirs for these viruses.

Viruses of the species *Mammalian 1 bornavirus* are distributed in central Europe in certain areas of Germany, Switzerland, Liechtenstein (BoDV-1), and Austria (BoDV-1/2) (47). These viruses are transmitted by bicolored white-toothed shrews (*Crocidura leucodon*) (14, 48–51) to spillover hosts such as horses, sheep, and captive New World camelids. In such hosts, these viruses can cause meningoencephalitides-associated central nervous system disorders and death (37, 52–54). Rarely, domestic rabbits, cattle, or dogs may develop disease (54). Disease caused by BoDV-1 and BoDV-2 in humans have not been reported to date. In 2015, a novel, divergent mammalian bornavirus, variegated squirrel bornavirus 1 (VSBV-1), was reported in healthy variegated squirrels and three humans with fatal disease (55). The close genetic relationship of this virus to BoDV-1/2 hints at a European origin from an unknown source.

Birds of several species, such as parrots, canaries (*Serinus canaria f. domestica*), estrildid finches, geese, swans, and ducks can harbor bornaviruses (46, 56). Avian bornaviruses are distributed worldwide (57). In parrots and some other birds, such as canaries and geese, these viruses can cause proventricular dilatation disease (PDD), a disease that was first observed in imported psittaciform birds in the United States and Germany in the late 1970s. Since then, the disease has become widely distributed in captive-bred parrots and South American parakeets because of the widespread trade in companion birds (58). Despite the close association of pet birds and the persons keeping them, disease in pet bird owners and breeders has not been documented.

TABLE 2 Overview of bornaviral genes and proteins

Protein	Gene	Characteristics	Function	References
Nucleoprotein	N	Non-structural protein, two isoforms of 40 kD and 38 kD	• Component of viral ribonucleoprotein (vRNP); encapsidates viral RNA to form viral nucleocapsid • Prone to be integrated into genome of infected cells • Target for autoimmune responses	(20, 21, 22)
Accessory protein	X	Multifunctional nonstructural protein	• Regulates viral transcriptase activity and translation by binding to P protein; essential for viral replication cycle • Suppresses apoptosis	(23, 24, 38)
Phosphoprotein	P	Nonstructural protein, phosphorylated at serine residues	• Component of ribonucleoprotein • Activates transcription • Interacts with P, X, N, and L proteins	(25,121)
Matrix protein	M	14.5-kD, nonglycosylated matrix structural protein that forms noncovalently linked tetrameric subunits	• Associates with vRNP complex in host cell nucleus; may be involved in viral replication or transport of viral components	(26,122,123)
Glycoprotein	G	94-kD, full-length glycosylated structural protein (gp94) obtained after posttransitional modification of primary 57 kD translation product Type I membrane protein	• Cleaved by cellular proteases into two nonglycosylated fragments. Incorporated into infectious particles • Involved in bornavirus entry • Induces neutralizing antibodies	(4, 5, 124)
RNA-dependent RNA polymerase L	L	190-kD, protein formed after splicing of a small upstream ORF to large, 3′ ORF Phosphorylated by cellular kinases and interacts with P protein	• Transcription and replication • Component of vRNP	(18, 27)

Reptile bornaviruses are a recent addition to the family *Bornaviridae* (6, 44). To date, their epizootiology and possible epidemiology is unclear, and their diversity is probably vastly underestimated.

Incidence and Prevalence of Bornavirus Infections

Subclinical Infection Rate

Varying titers of antibornavirus antibodies have been detected in humans and other mammals worldwide without any link to a specific disease (59). These antibody titers are low in general and reflect different degrees of avidity (60, 61). Because bornaviruses are also widely distributed in birds of diverse species and species of other animal classes (reptiles), assessment of whether mammalian antibodies represent subclinical infections or simple exposure to non-mammalian bornaviral antigens is difficult. The higher prevalence of antibornavirus antibody-positive horses living along flight paths of migratory birds supports the latter hypothesis (62). Animals that recover from bornavirus infections usually have high long-term antibody titers (63, 64). Therefore, low antibody titers in apparently healthy mammals of numerous species are difficult to explain and cannot be differentiated from nonspecific cross-reactivities (e.g., as a result of epitope similarities of antibodies after infection with still unknown viruses distantly related to bornaviruses or imprints into the immunological memory once persistent infection had been overcome in the evolutionary past in species harboring EBLNs). Interestingly, the antibody titer in a recently reported fatal human case was extremely high and was clearly connected to the detection of high genome copy numbers of the novel bornavirus from variegated squirrels, VSBV-1 (55).

Thus, the usefulness of antibody detection in diagnostic techniques is limited by two epidemiological factors: 1) the wide distribution of mammalian, avian, and most probably other bornaviruses that may broadly cross-react with most of the existing serological assays (3), and 2) EBLNs in genomes of mammals of several species that could result in the expression of proteins that interfere with enzyme-linked immunosorbent assays (ELISA) using monoclonal antibodies against epitopes of the N protein (Dürrwald, unpublished observation).

Zoonotic Epidemic Patterns

The frequency of Borna disease in horses varies annually (47), which may reflect population fluctuations of the transmitting virus reservoir, bicolored white-toothed shrews (14). Infections of humans have not been reported within this epidemiological chain. Zoonotic epidemic patterns have not been described for PDD caused by avian bornaviruses.

Three patients infected by VSBV-1 from captive variegated squirrels developed fatal encephalitis in 2011 and 2013 (55). The variegated squirrels harbored VSBV-1 in all organs, similar to that reported for persistently infected, immunotolerant animals. Very high genome copy numbers were detected especially in the central nervous system (55). Virus was also detected in oropharyngeal swabs (55). However, the number of cases of human VSBV-1 infection is too low to establish a clear epidemiologic relationship.

The three fatal human cases of VSBV-1 infection provided evidence that bornavirus infection can lead to disease characterized by clinical signs similar to animal Borna disease (55). The question arises as to why such fatal human infections have not been observed in the past with other mammalian bornaviruses. Two major explanations are possible: 1) the transmission risk for humans was higher in the captive variegated-squirrel-to-human transmission route than in the bicolored-shrew-to-horses infection chain (Figure 3), or 2) VSBV-1 and BoDV-1/2 differ in their virulence. Direct contact of farmers with free-living shrews is very rare. Bites and scratches could have occurred during the handling of persistently infected laboratory animals. However, despite the long history of BoDV-1 research, no confirmed cases of Borna disease have been reported in laboratory staff.

Reinfections

The issue of reinfections has not been investigated. Infected cells are resistant to superinfection (65), which may be caused by EBLNs inhibition of infection by related exogenous viruses (11).

Seasonality of Borna Disease

Borna disease in horses, sheep, and other spillover hosts peaks in late spring and early summer and declines to a nadir in the fall (47). Such seasonality probably results from exposure to bicolored white-toothed shrews in combination with the long incubation period in the spillover host (14). Infections and PDD in parrots and other birds are not seasonal (46, 56, 58). Seasonality was not observed for VSBV-1-induced human encephalitis (disease onset in November and June) (55).

Transmission of Bornaviruses

Routes

The transmission of bornaviruses of the species *Mammalian 1 bornavirus* is unknown. Persistently infected bicolored white-toothed shrews carry bornaviruses in the bladder and keratinocytes in the skin, which may lead to environmental contamination (dust) from urination and skin sloughing (14, 48, 50, 51). The bulbus olfactorius in infected horses and other spillover hosts is a postulated site of entry, from where bornaviruses travel to the central nervous system (66, 67). Transmission of VSBV-1 to humans may have occurred by scratches and bites (55). Fecal-oral and aerogenic feather dust transmission routes have been hypothesized for avian bornaviruses (46, 56).

As suggested by infection of keratinocytes in bicolored white-toothed shrews (BoDV-1) (14) and feather follicles in birds (avian bornaviruses) (68) and the irregular transmission of avian bornaviruses after experimental contact exposure (68), parasites such as mites may be virus vectors. *In vitro* experiments revealed that BoDV-1 can survive in ticks for a month (69). The role of vectors in bornavirus transmission has not yet been investigated.

Risk Factors for Transmission

The risk factor for transmission of bornaviruses is exposure to reservoir hosts. At the time of writing, only three cases of human bornavirus infection have been unambiguously identified (55). Consequently, risk factors for transmission of bornaviruses to humans are still undefined. The three infected humans had close contact with variegated squirrels infected with VSBV-1, but the infection route is not yet known (55).

EBLNs more closely related to extant bornaviruses were found in 13-lined ground squirrels, which, like variegated squirrels, also live in America. American squirrels should thus be regarded as a possible source of bornaviruses, some of which may be pathogenic for humans. Close contact with bicolored white-toothed shrews and variegated squirrels kept in zoos and private collections should be avoided unless they have been tested and are free of bornaviruses. Wild animals of these species should not be handled without protective wear. Risk of human-to-human transmission after specific exposures (e.g., household, needle stick, transfusion, sexual contact, perinatal) has not been investigated to date. The risk of nosocomial infection (hospital, nursing home) is not known. Likewise, the susceptibility of humans to bornavirus infection has not been investigated.

For horses and other spillover hosts, contact with bicolored white-toothed shrews and possibly other hosts yet to be discovered is a risk factor for infection with viruses of the species *Mammalian 1 bornavirus*. Indeed, defined endemic regions of Borna disease correlate in some instances with areas of known distribution of bicolored white-toothed shrews (14). Transmission occurs more frequently when these shrews enter stables in the fall, again emphasizing the importance of proximity to these shrews in the epizootiology of animal Borna disease.

A risk factor for parrots and other birds in developing PDD is the exposure to other parrots/birds infected with viruses of the species *Psittaciform 1 bornavirus* or other avian bornaviruses not yet classified. Infected parrots shed virus through feces and probably feather dust. The exact transmission route for avian bornaviruses is still unknown. Therefore, additional risk factors may be involved.

PATHOGENESIS OF BORNA DISEASE IN ANIMALS

Two major factors are important in pathogenesis: 1) infection of the central nervous system by bornaviruses, and 2) immunopathology reflected by a strong $CD8^+$ T-cell response to the bornavirus N antigen of infected cells, which do not undergo apoptosis after infection.

Incubation Period

The incubation period for Borna disease in horses is not known exactly. From study of horses that were exported from endemic areas to nonendemic areas where they developed the disease, the incubation period is estimated to be 5 ± 3 months (14). In parrots, the incubation period of PDD ranges from half a month in unweaned chicks to more than 5 months in adult parrots with natural and experimental infections (70, 71).

Patterns of Virus Replication

BoDV-1 in animals with Borna disease is found mainly in the brain, the spinal cord, and neurons (37, 39, 72). In horses and other mammals, including experimentally infected rhesus monkeys, BoDV-1 replicates to high titers in the retina (37, 73). BoDV-1 is detected in horses irregularly in conjunctival fluid, nasal secretions, and saliva (39, 74).

After experimental intranasal infection in rats, the main small animal model for Borna disease, BoDV-1-specific antigen can be first identified in neuroreceptors of the olfactory epithelium and later in the brain (75). The virus replicates in neurons, ependymal cells, astrocytes, and oligodendrocytes but not in vascular endothelial cells (75). In adult immunocompetent rats that survive BoDV-1 infection,

BoDV-1-specific antigen is found in cells of the peripheral nervous system (e.g., Schwann cells) and axons of peripheral neurons (75).

The time course of infection in natural spillover hosts is not known. In rats experimentally infected with BoDV-1, signs of encephalitis peak around day 21 after inoculation and begin to decrease in surviving rats 30 days after inoculation. Inflammation is almost absent, and neuronal cell loss peaks at 70 days after inoculation (75).

Avian bornavirus-infected adult birds can develop severe lymphoplasmacytic inflammation in peripheral, central, and autonomic nervous tissues associated with gastrointestinal and neuronal signs. Also, destruction of Purkinje cells in the cerebellum is observed (56). Avian bornaviruses are distributed in many tissues (71). Thus, birds reflect characteristics of bornavirus infection in newborn mammals (wide tissue distribution of virus and virus shedding) but also of infection in adult mammals, such as inflammation. The effects of avian bornavirus infections have not yet been investigated in newly hatched birds.

Factors in Disease Production

Acute BoDV-1 infection is similar in horses, sheep, and experimentally infected laboratory animals (e.g., rats). Inclusion bodies are detected in the nuclei of infected cells (75). Inflammation in rats is centered in the limbic system but also spreads to other parts of the brain. In surviving rats, inflammation decreases and disappears after 2 to 3 months despite ongoing virus replication, but a dramatic loss of neuronal tissue and severe hydrocephalus occurs.

Immune Responses

The immunological response to BoDV-1 infection is best characterized in experimentally infected rats and mice. BoDV-1 infection activates T and B lymphocytes. In infected rats, disease is associated with a mononuclear inflammatory reaction in the central nervous system with severe perivascular cuffing of T cells, macrophages, and also B cells during later infection (75). $CD4^+$ T helper cells and $CD8^+$ cytotoxic or suppressor cells are detected after inoculation of rats (76–79). $CD8^+$ T cells play a major role in immunopathogenesis in experimentally infected rats and mice (80). Consequently, BoDV-1-induced disease is defined as a $CD4^+$ T-cell-dependent immunopathological process mediated by CD8+ T cells (81). Elevated concentrations of proinflammatory cytokines (IL-6, IL-2, IL-4, TNF-α, IL-1α, IL-1β, TGF-β1, IFN-γ) and chemokines (IP-10, RANTES = CCL5) as well as chemokine regulators (COX-2, CGRP, iNOS) have been measured in the brains of neonatal and/or adult rats experimentally infected with BoDV-1 (75).

In mammals with Borna disease and parrots with PDD, immune cells infiltrate into the central nervous system. In mammals, $CD8^+$ T cells attack infected cells and cause meningoencephalitis or lymphoplasmacytic infiltration in other parts of the central nervous system. Immunopathology in birds has not yet been investigated in detail.

Antibodies, predominantly against N and P proteins, occur approximately 7 days after experimental BoDV-1 inoculation in infected rats (75). Persistently infected immunotolerant rats and surviving rats or rabbits have high titers of antibodies (37, 63, 82). Horses naturally infected with BoDV-1 have low antibody titers, which can increase shortly before death (83). This increase may reflect humoral responses that come too late in the disease course. Parrots develop high titers of antibodies detected by immunofluorescence within the first 12 weeks after experimental infection with avian bornaviruses. These titers remain stable at a high plateau (71). Recurrent infections are not known. Neutralizing antibodies that occur with decline or lack of symptoms can serve as a correlate of disease resolution.

Of the three human patients infected with VSBV-1, one was investigated for the presence of virus-specific IgG antibodies by an indirect immunofluorescence antibody test. Very high antibody titers were measured in cerebrospinal fluid (1:2560) and serum (1:5120) (55).

CLINICAL MANIFESTATIONS IN HUMANS

For the first time in 2015, three fatal cases of bornavirus-induced encephalitis in humans were reported in the state of Saxony-Anhalt, Germany. The first clinical case was seen in 2011 and the second and the third in 2013 in different hospitals (55). Affected persons were 62- to 72-year-old males with age-typical health status. Each of them was known to breed variegated squirrels, rodents common to Central America that came into fashion as exotic outside pets during the last 15 years. The three breeders knew each other but did not live in close proximity to one another; however, they exchanged animals (55).

Symptoms

During the prodromal phase, the patients presented with fever and rigors, fatigue, weakness, and walking difficulties (55). Because of increased confusion and psychomotor impairment, the patients were admitted to neurology wards where they continued to deteriorate. Myoclonus, opsoclonus or ocular paresis, sopor, and coma were typical clinical signs, and tetraparesis, divergent bulbi, and grimacing were observed in one patient. Bilateral crural-vein thrombosis leading to pulmonary embolism was also observed. Encephalographic findings reflected mainly delta activity but also short alpha episodes and low-voltage theta or theta-like activity in individual patients. Treatment with antibiotics and glucocorticoids had no discernible effect. All three patients died, most probably as a result of progressive encephalitis, despite intensive care and mechanical ventilation. The time from onset of first symptoms to death ranged from 2 to 4 months.

Further molecular and immunohistological analysis of brain tissue from the three deceased patients confirmed presence of a novel bornavirus, VSBV-1, which is only 77% identical to BoDV-1, indicating that VSBV-1 may require classification as a novel bornavirus species (55). Importantly, the clinical signs of the three fatal cases in humans resemble those seen in horses with Borna disease described below. Other clinical courses with this novel virus, the time course of infection in humans, and disease presentation in immunocompromised patients and at different ages are unknown. The possibility of subclinical infections with BoDV-1 and other bornaviruses cannot be addressed because of limitations in current diagnostic techniques.

Clinical Diagnosis, Including Differential Diagnosis

Confusion, psychomotor impairment, and weakness could be suggestive of the clinical diagnosis. Depending on the region, viral infections such as rabies should be considered in differential diagnosis. Other nerve disorders such as epilepsy may be taken into account in relation to the degree of seizures observed. Parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis, Huntington's disease, and

Alzheimer's disease may be excluded because of their more extensive chronic course.

Psychiatric Disease and Borna Disease Virus Infection

Because of the neurological character of Borna disease in horses, primary research activities have been directed to patients with neurological disorders. Soon after the establishment of the first immunofluorescent antibody assays, bornavirus-specific antibodies were detected in psychiatric patients (84). Based on these findings, a hypothesis of the involvement of BoDV-1 in mood disorders was formulated and followed for more than a decade (85). The focus on mood disorders was difficult to understand because antibodies were also found in other patient groups and at higher prevalence in comparison to psychiatric patients (86). Later, genetic evidence of bornavirus infection was reported in psychiatric patients worldwide (59). Meta-analyses revealed that all sequences of bornaviruses in humans reported so far were identical to commonly used laboratory strains (87, 88). Later, researchers found that antibodies to BoDV-1 do not correlate with bornavirus infection in patients with schizophrenia, bipolar disorder, or major depressive disorder (89).

Comparative Analysis of Clinical Features of Bornavirus Infection in Animals

So far, two major disease complexes caused by bornavirus infections are known in immunocompetent adult animals, Borna disease in mammals and PDD in parrots. Meningoencephalitis (mostly fatal) in horses is reflected clinically by increasing apathy, purposeless circling, inability to achieve correct leg positioning, and gulping or, in some cases, paralysis (90, 91).

In parrots, inflammation of peripheral nerves causes dysfunction of the proventriculus as reflected by regurgitation, passing undigested seeds, and weight loss (58). Neurological symptoms, such as ataxia or seizures, occur in some cases. In contrast to Borna disease in mammals, which lasts some weeks before death occurs (54), PDD can last for months and can also become chronic (58).

Newborn mammals that become infected with BoDV-1 mostly become immunotolerant because their $CD8^+$ T cells do not recognize bornavirus antigen (92). In these animals, virus is distributed throughout all tissues despite the presence of high titers of antibodies, and the virus is shed in large amounts in (75). Despite immunotolerance, the high viral burden can cause astrocytosis, microgliosis, and/or loss of Purkinje cells associated with discrete brain damage. Nonfatal clinical signs, such as learning deficiencies or stagnation in weight gain are observed (75, 93, 94) (Figure 2).

Genetic factors may play a great role in induction of bornavirus infection of both mammals and birds. Variation in disease outcome is high between animals of different species, but also between individuals of the same species (Figure 2). In horses, subclinical infections with recovery were reported in a few cases (52). Serological and virological data support the occurrence of subclinical infections in

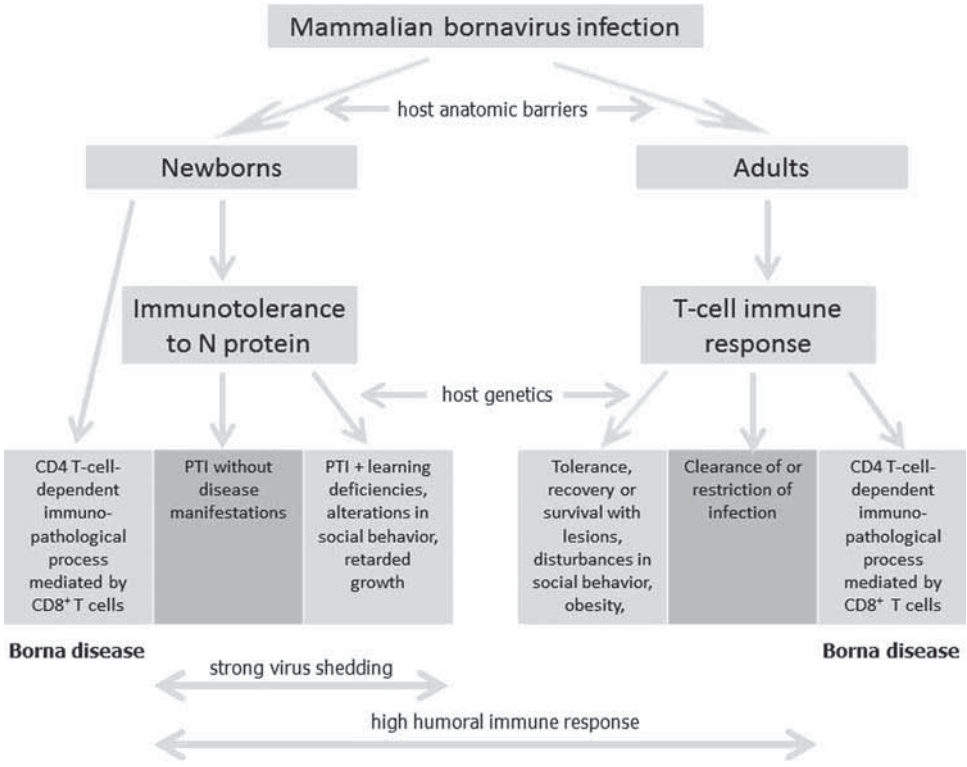

FIGURE 2 Outcome of bornavirus infections and pathogenesis in mammals. The prevalence of natural disease is low and varies among hosts. Differences in anatomic or other barriers and transmission routes may exist that enable or prevent entry of bornaviruses into the CNS. A wide variety of outcomes of experimental and natural infection is probably the result of host genetic factors. PTI, persistent tolerant infection.

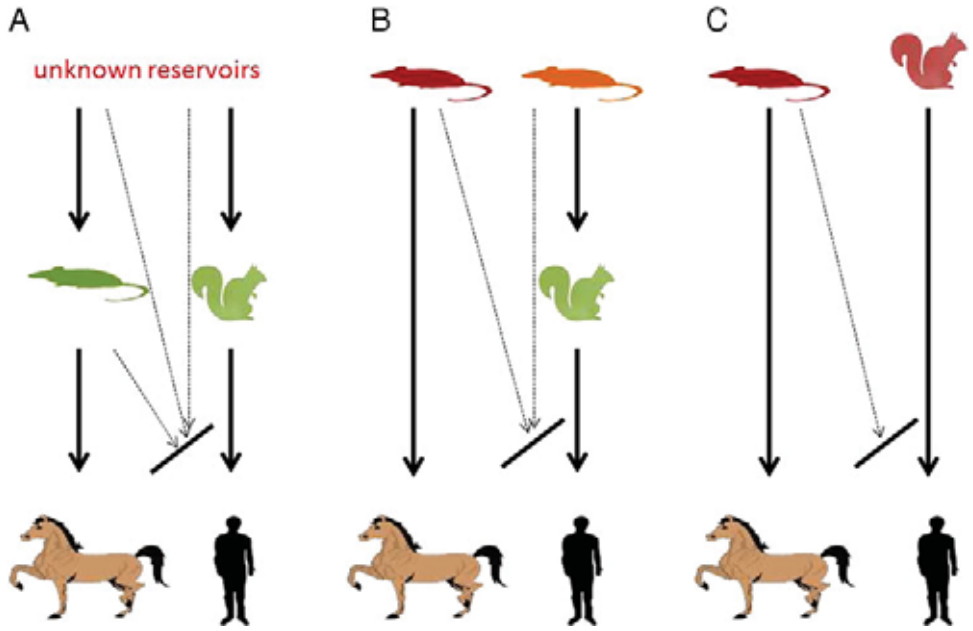

FIGURE 3 Hypothetical transmission scenarios. A, Bicolored white-toothed shrews and variegated squirrels are intermediate hosts to an unknown reservoir. B, Different shrew species are reservoir hosts and variegated squirrels are intermediate hosts. C, Bicolored white-toothed shrews and variegated squirrels are the primary reservoir host. The exact transmission route is unknown. The strong territorial genetic clustering of BoDV-1 sequences and the high conservation within BoDV-1 group 4 (strain V group) over a vast territory hint at transmission chains that are more complex than shown in this scheme (125). Natural barriers may block transmission from the primary reservoir to humans. Close contact with intermediate hosts may enable infection of the nerve cells of humans under certain circumstances (scratches, bites?). Note that scenarios 1 and 2 require constant surveillance of intermediate host populations when kept in captivity (zoological gardens, private collections). It is still unknown whether bicolored white-toothed shrews and variegated squirrels were infected as newborns or adults. The slanted line means that transmission does not occur.

horses (54). In birds, the number of subclinical infections is high (68, 71).

The major difference in infection with BoDV-1/2 of mammals at different ages is the immunotolerance observed in experimentally infected newborn laboratory animals. For all other ages, no differences in presentation of Borna disease have been observed. In parrots, PDD is more severe in unweaned birds (70).

Splenectomized rhesus monkeys experimentally infected with BoDV-1 developed fewer clinical signs and less histopathology than control animals (73). Athymic or immunocompromised rats that were experimentally infected expressed no or fewer signs of disease and inflammation than did immunocompetent rats (95–97).

LABORATORY DIAGNOSIS

Post-mortem diagnosis is still the most reliable diagnosis for mammals naturally infected with BoDV-1 because of irregular shedding and detection of bornaviral markers in tissues and cells (e.g., peripheral blood mononuclear cells) and of the wide distribution of low-titer antibodies in the healthy population (98). In persistently BoDV-1-infected-mammals and birds infected with avian bornaviruses, a broad spectrum of methods can be applied for *intra-vitam* and *post-mortem* diagnoses (56, 98).

Virus Isolation

Brain samples are best suited for virus isolation (56, 98). Other organ samples from experimentally persistently infected animals and infected birds can be used for virus isolation (37, 56).

Primary brain cells of newborn rabbits were used successfully for isolation of mammalian bornaviruses. Permanent cell lines, such as oligodendroglial (OL) cells, MDCK, RK-13, and many other mammalian and bird cells, are suited for isolating mammalian bornaviruses (56, 98). The viruses do not cause a cytopathic effect. Infected cells can be detected by immunostaining (36, 37). Avian bornaviruses replicate mainly in avian cells (quail cells such as CEC-32, QM7; chicken fibroblasts DF-1; primary duck embryo fibroblasts) and are difficult to isolate in mammalian cells (56). VSBV-1 is not isolated at the time of this writing.

Antigen Detection

Bornavirus antigen detection is possible via polyclonal antisera and monoclonal antibodies (98). Specific antibodies can be used for staining of brain and other organs (immunohistochemistry), and of organ homogenates (ELISA and other techniques, such as Western blot).

Nucleic Acid Detection

Bornaviral nucleic acid detection is possible by reverse transcriptase polymerase chain reaction (RT-PCR). Many RT-PCR protocols are available (69, 70, 98). For the detection of VSBV-1 special real-time RT-PCR-protocols exist (55). Nucleic acid detection is also possible by *in situ* hybridization in organ and brain samples.

Serological Assays

Detection of bornavirus-specific antibodies is routinely performed by serological assays, such as indirect fluorescent antibody test, ELISA, or Western blot (98). The broad cross-reactivity between different bornaviruses should be taken into consideration when diagnostic techniques are applied. Because positive antibody reactions could be caused by different viruses, a virus species-specific diagnosis via the described serological methods is not possible. In contrast, antibodies directed against the glycoprotein (G) do not cross-react between different bornaviruses (3). Neutralization assays are also used (36, 82).

PREVENTION

Human Bornavirus Infections

Individuals may be at an increased risk of exposure to bornaviruses during handling of persistently infected, immunotolerant animals. Standard precautions, such as donning proper personal protective equipment (PPE, such as gloves, protective eyewear) have been sufficient when handling mammalian or avian bornaviruses. However, rigorous infection control precautions should be taken when handling VSBV-1. The risk of human infections transmitted from avian bornaviruses is most likely lower compared to mammalian bornavirus because of the poorer replication of avian bornaviruses in mammalian cells. In general, procedures that increase the risk of bites from infected laboratory animals or injuries with sharps should be performed following strict safety procedures.

Nothing is known about human-to-human transmission of bornaviruses. From the three human cases, no evidence for any human-to-human transmission could be seen, and the risk might be negligible. Comparative analyses of borna disease and rabies in animals have been done (99). Experts advise staff to take the same precautions with bornavirus-infected patients as with patients infected with rabies virus until bornavirus infection and transmission in humans has been investigated in more detail. Antiviral chemoprophylaxis has not yet been investigated.

Infections occur sporadically. It is likely that many bornaviruses have yet to be discovered and that some may be pathogenic for humans. If a bornavirus infection is diagnosed in patients, possible contacts with exotic animals should be investigated, followed by proper risk analysis and containment responses.

Animal Borna Disease

Passive Immunoprophylaxis

Passive immunoprophylaxis has not yet been investigated. Neutralizing antibodies can prevent encephalitis in experimentally BoDV-1-infected animals (5, 82). Neutralizing antibodies can be induced in rabbits at high titers after high-dose inoculation with cell-cultured virus (63).

Active Immunization

Human bornavirus vaccines are not yet available, but studies of experimental vaccines in animal models have been performed. Active immunization with live vaccines established by serial passage of BoDV-1 in rabbits was common in horses and sheep in Borna disease-endemic areas of Germany from the 1920s until the 1990s (100). Vaccination was terminated because the risks of virus distribution could not be assessed (100). The induction of transient low-titer bornavirus neutralizing antibodies in inoculated horses after vaccination does not support the establishment of vaccine-induced infection in vaccinated animals (100).

All other vaccine approaches are thus far experimental in nature. High doses of active cell-cultured BoDV-1 induced humoral and cellular immune responses that protected rats and rabbits after intracerebral inoculation (63, 101, 102). However, low doses were fatal, most probably as a result of the dilution of cell components contained in the inoculum. Recombinant vaccines expressing BoDV-1 N protein ensured survival of rats (103, 104). BoDV-1 N protein is a major target for the $CD8^+$ T-cell response (105). Therefore, the disease can be aggravated if infected rats are vaccinated with N protein (106).

TREATMENT

Antiinflammatory treatment is the major way to interrupt immunopathology in animals. Treatment of experimentally BoDV-1-infected rats with TGF-β2 only transient reduced clinical signs but a significant decrease in inflammation in the brain (107). Treatment with anti-$CD8^+$ monoclonal antibodies reduced or inhibited inflammation and prevented neuronal degeneration (108). Treatment with cyclophosphamide or cyclosporine prevented or reduced inflammation and disease (96, 97).

Amantadine did not affect BoDV-1 *in vivo* and *in vitro* (109–111). Ribavirin inhibited transcription and replication in cell lines (112, 113) but did not affect viral load in experimentally infected rats. However, treated rats had less inflammation and developed milder clinical signs perhaps related to ribavirin's immunomodulatory effects (114). Also, 1-β-D-arabinofuranosylcytosine inhibited BoDV-1 replication in cultured cells and in experimentally infected rats and reduced clinical signs (41, 115, 116); 2′-fluoro-2′-deoxycytidine inhibited BoDV-1 replication and spread (117). BoDV-1 is sensitive to type I IFN (interferon) in cell culture (118). IFN-α and IFN-β inhibited replication but promoted transcription of BoDV-1 in persistently infected cells (119). However, BoDV-1 is able to prevent type I IFN induction by trimming the 5′ ends of its genomic and antigenomic RNA, thereby removing triphosphorylated residues. Retinoic-acid-inducible gene (RIG)-1, which is essential for the induction of IFN, cannot sense such nontriphosphorylated 5′ termini (120).

No treatments of proven value in naturally infected animals are available. As a consequence of the pathogenesis of bornavirus infections, two factors are important to control disease that should be the focus of further research: 1) prevention of central nervous system infection (prophylactic and postexposure vaccination) and 2), prevention of immunopathology once infection has occurred (specific anti-inflammatory and antiviral treatments).

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Hepatitis D

MARIO RIZZETTO, ANTONINA SMEDILE, AND ALESSIA CIANCIO

58

Hepatitis D virus (HDV) is unique in animal virology and pathology. It has a circular RNA genome of the smallest size among human viruses, requires the hepatitis B surface antigen (HBsAg) capsid provided by the hepatitis B virus (HBV) to assemble into infectious virions, parasitizes the transcriptional machinery of the host by hijacking cellular RNA polymerases to replicate its RNA genome, and is replicated by a rolling circle mechanism unknown in mammalian cells.

HDV is a defective RNA virus recognized in the late 1970s as a new cause of hepatitis. Its discovery followed the description in Torino of a new antigen, named delta, in the liver of patients with chronic HBV liver disease (1). Subsequent studies at the National Institution of Health US revealed that the delta antigen was the expression of a new hepatitis virus, which was named HDV. The term delta virus and delta hepatitis are still used as synonyms of HDV and hepatitis D. Cloning and sequencing of the viral genome in 1986 established the peculiar features that make HDV unique among animal viruses (2, 3). The host range of HDV infections includes humans, chimpanzees, and woodchucks carrying the woodchuck hepatitis virus (4).

Infection is present worldwide (5). Carriers of the HBsAg superinfected by HDV are the major victims of the infection and the reservoir of the virus. HDV infection almost invariably results in liver damage, often a severe and progressive hepatitis conducive to cirrhosis (6), and is the most severe form of chronic viral liver disorders. Vaccination against the HBV has reduced the spread of HDV in industrialized countries over the last 20 years. However, hepatitis D is returning to Western Europe through immigration from regions where HDV remains endemic and is being rediscovered in the developing world, where it has a major medical impact in many areas of Africa, Asia, and South America. Therapy against HDV raises a formidable problem because the virus has no replicative function of its own to be targeted. Empirical pegylated-interferon (peg-IFN) treatment remains the mainstay of treatment. New strategies aimed at blocking the entry of the virus and its assembly into virions are being explored.

VIROLOGY

The HDV is the only member of the family Deltaviridae, genus *Deltavirus* (7). To establish natural infection, HDV requires the HBsAg provided by the HBV (8). The virion is a chimera composed of the HDV RNA genome and protein, both enveloped by the HBsAg (Fig. 1). Eight genotypes with different geographic distributions have been identified by comparative phylogenetic analysis (9). Their lengths fall in a narrow range, from 1672 to 1697 nucleotides, with 81–89% homology in nucleotide sequences within the same genotype and as much as 35% divergence between different genotypes.

Genome Structure

The genome consists of a circular, single minus-strand RNA (2, 3). The HDV particle varies between 35 and 41 nm in diameter, with no defined nuclocapsid structure (10, 11). The genome is self-complementary and folds into an unbranched rod structure with base pairing of about 70% of the nucleotides. Three major RNA species are found in humans (Fig. 2) (8, 12). The first is the 1.7-kb genomic RNA contained in the virions; the second is the complementary antigenomic RNA of positive polarity, present in the liver; and the third is a shorter polyadenylated messenger RNA (mRNA) of 0.8 kb found in the liver. The numbers of genome, antigenome, and mRNA are estimated at 300,000, 60,000, and 600 copies, respectively, per infected cell.

HDV replicates through a unique rolling circle mechanism (8, 12). Crucial to replication is the presence of a ribozyme less than 100 nucleotides in length, conferring autocatalytic capacity (self-cleavage and self-ligation) for both the genomic and antigenomic strands without the need of other enzymatic functions (13–15). It is the fastest, naturally occurring, self-cleaving RNA required for viability in a human pathogen, but differs from the "hammerhead ribozymes" described in plant viruses. The HDV ribozyme has been crystallized (16).

Replication Cycle

The sodium taurocholate cotransporting polypeptide (NTCP) is the entry receptor for HBV and HDV (17). Glypican 5 is a host cell entry factor common to both (18). The pre-S1 region of the large HBsAg is essential for entry (19); monoclonal antibodies to the pre-S1 domain block HDV infection in mice expressing the human NTCP. Entry is inhibited by cyclosporin A (20), by primary bile acids (21), and by vanitaracin A, a tricyclic polyketide (22).

After entry into hepatocytes, genomic HDV RNA is transported to the nucleus where it replicates into the antigenome by a rolling circle mechanism. A nuclear localization

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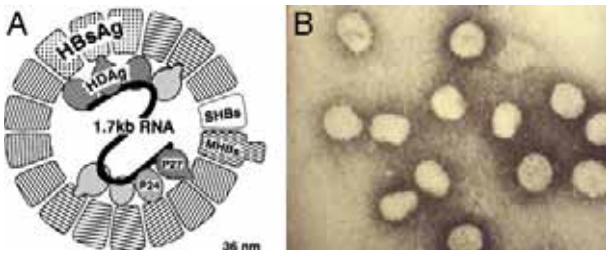

FIGURE 1 A: Schematic representation of HDV B: The HDV particle in electron microscopy.

signal mediates the initial step (23). The rolling circle mechanism involves transcription over the circular genomic RNA of multimeric linear transcripts of antigenomic sense that undergo autocatalytic cleavage by the ribozyme and are then ligated by the ribozyme and probably by a host ligase, with the final production of circular antigenomic RNA (12, 15). The antigenomic RNA serves as a template for replication of the circular genomic RNA by similar transcription and processing (Fig. 3). HDV has no known enzymatic capabilities; the viral genome is replicated primarily by host DNA-dependent RNA polymerase II (24); host RNA polymerase I and II are also involved to a lesser extent. The features of the HDV are summarized in table 1.

HDV Proteins

The 0.8-kb mRNA is transcribed from the antigenome (12, 15). This species has a 3′-polyadenylated tail and a unique 5′ end with a specific site for the initiation of transcription (15). The mRNA contains the only open reading frame of the HDV, which is translated into the HD antigen (HDAg) existing in two forms—the small 24-kDa HDAg (s-HDAg) and the large 27-kDa HDAg (l-HD Ag) (25). The HDAg is located predominantly in the host nuclei and may also be localized in nucleoli (26). With increasing levels of l-HDAg expression, the intracellular distribution includes cytoplasmic localization.

The earliest translated product, s-HDAg, contains 195 amino acids, is essential for replication, and promotes the

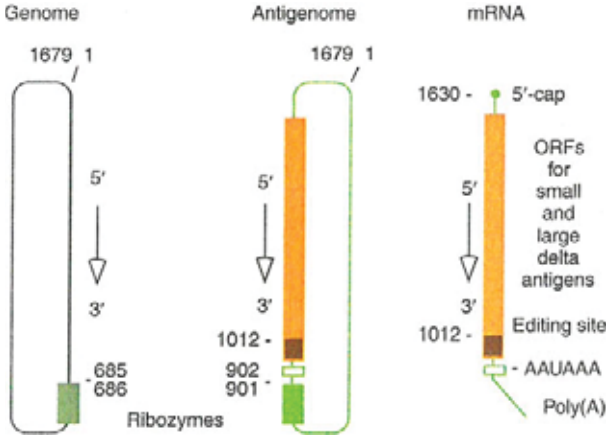

FIGURE 2 Circular HDV-RNA genome and antigenome (unbranched rodlike structures). Rybozime (solid green box). Open reading frames for the small and large HD antigens (solid brown box). Short linear messenger RNA(mRNA) containing the AAUAAA polyadenylation signal and the editing site. (Reprinted with permission from reference [15].)

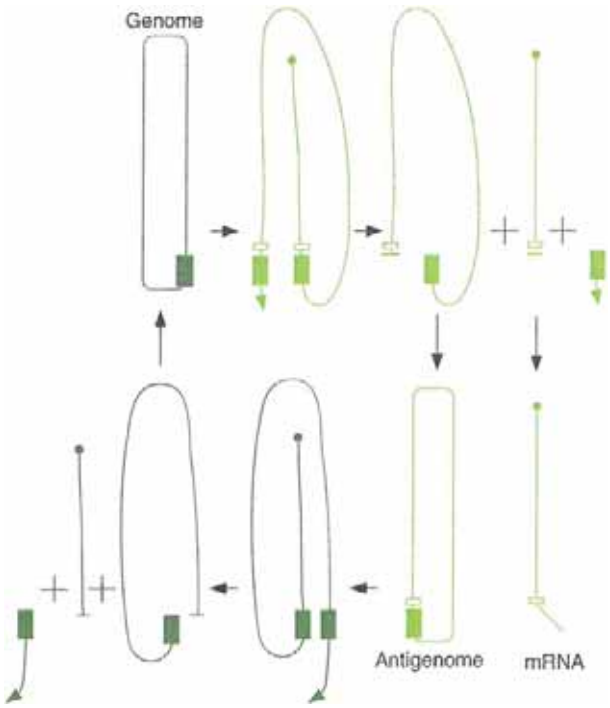

FIGURE 3 Rolling-model for the replication of the HDV-RNA genome. Redirection of host polymerases produce multimeric transcripts of HDV-RNA which undergo self cleavage and ligation to unit-length circular RNAs through the catalysis of the HDV ribozymes (filled rectangles). (Reprinted with permission from reference [15].)

accumulation of the viral RNA in cells. It might facilitate the transcription of HDV RNA by RNA polymerase II. During replication, the s-HDAg is elongated on the same reading frame to the l-HDAg through an editing process mediated by a host double-stranded RNA adenosine deaminase (25), whereby the amber UAG stop codon terminating the s-HDAg is converted to a UGG codon for tryptophan, which leads to the translation of 19 additional amino acids with the final 214-amino-acid length of the l-HDAg. The l-HDAg is a dominant negative inhibitor of HDV RNA replication and is essential for the assembly of the HD virion.

The two HDAg isoforms are essential to the biology of HDV. Various posttranslational modifications of both isoforms (27), including phosphorylation, methylation (28), acetylation, and sumoylation (29), can change the functions of the HDAg, presumably providing molecular switches that orchestrate the sequence of the various steps involved in the replication cycle of HDV (27, 30). Critical to virion assembly and export is the farnesylation (prenylation) of the cysteine in the carboxyl terminus of the l-HDAg (31, 32), the so-called cxxx box, which contains a cysteine (c) as the fourth amino acid from the terminus; the three terminal amino acids are removed in the farnesylation process. This modification contributes to the ability of the l-HDAg to inhibit replication and is critical to drive the l-HDAg to combine with the HBsAg to assemble the virion (15).

HDV-HBV Interactions

The small HBsAg protein (s-HBsAg) is sufficient for assembly of the HDV particle, but infectious particles are produced only if the large HBsAg (l-HBsAg) is present (33, 34). Both HDAgs connect to each other and to HDV

TABLE 1 Features of Hepatitis D virus

Smallest infectious agent in humans, 1700 nucleotides
Circular, single-stranded, negative polarity
Infectious at 10^{-11} serum dilutions in hepatitis B surface antigen
Rolling circle mechanism of replication
Self-cleaving ribozyme
Transcription by host-RNA polymerases

RNA through RNA-binding domains to assemble the HDV ribonucleoprotein (RNP) (35). The envelopment of HDV occurs through interactions of HBsAg proteins with the RNP (15, 35). The ability of the HDAgs to bind the nucleic acid is largely controlled by their ability to multimerize (36).

Most of the sites binding the HDAg are located on the s domain of the HBsAg, with some hot spots for the envelopment process; their integrity is critical to virion formation (37). The s-HBsAg residues 24–28 and 56–80 are important for HDV packaging and secretion. Also important are tryptophan residues at positions 196–199 and 201 of the carboxy-terminal peptide (38). The interactions of HDAg with s-HBsAg, which are necessary for virion assembly, are ablated by the substitutions Phe196Trp, Phe199Trp, or Phe201Trp in the s-HBsAg.

Mutations or conformational changes in cysteine residues of the antigenic loop of the HBsAg (39), which contains the conserved "a" determinant involved in HBV infectivity, block the entry of HDV into cells. HBV plays a role in HDV infectivity; sequences of natural HBV variants can influence the assembly and secretion of HDV (40). The specific infectivity of HDV particles varies 160-fold with HBV envelope proteins of various genotypes, with a decreasing trend in infectivity from genotype D through genotypes B, E, and A (41). In human hepatocellular-derived cell lines, small and large HBsAg proteins produced by HBV-DNA sequences that are naturally integrated during infection support the formation of HD virions in the absence of HBV virions or replication (42).

HBV

Mutants generated by point mutations, small insertions, or deletions at the tip of the genomic rod structure may affect HDV replication by reducing its efficiency 100- to 1000-fold compared to the wild type (43). Substantial variations between the structure of the RNA editing sites of the different genotypes may impact the efficiency or mechanisms of editing. HDAg binds to the clamp of RNA polymerase II and affects its structure and conformation (44).

A feature of HDV infection is the inhibition of HBV-DNA synthesis, whereas the HBsAg necessary for HDV assembly is not diminished. HDV nucleoproteins competing with HBV for the HBsAg induce a selective suppression of HBV replication associated with an increase in pre s-s RNAs and in the levels of HBsAg (45). The HDAgs inhibit HBV-DNA synthesis through the suppression of HBV enhancers and the transactivation of the alpha-inducible myxovirus resistance A (MxA) gene (46).

HDV Monoinfection and Latency

HDV needs only the capsid of HBsAg to enter hepatocytes. It is replicated inside nuclei by host RNA polymerases without help from HBV. Monoinfection and latency of HDV in the absence of HBV have been demonstrated *in vitro* and *in vivo*. HDV RNA synthesized *in vitro* induces helper-independent genome replication in cultured cells (47). HDV monoinfection was initiated by the injection of HDV complementary DNA (cDNA) clones in the liver of transgenic mice not susceptible to HBV infection (48), with the production of replicative intermediates of HDV and expression of HDAg in the liver in the absence of HBV markers. Transgenic mice can support independent HDV RNA replication in multiple tissues, in particular in skeletal muscle (49). The mouse model is now used for the study of HBV and HDV interactions and for preclinical drug evaluation (50).

HDV replication in humans, apparently independent from HBV, has been reported in liver transplants (51–53). The HDAg remained detectable without HBV weeks to several months in the liver of transplanted patients protected with immunoglobulins against the HBsAg. The latent HDV infection was not accompanied by disease. Liver damage developed only when the underlying HDV was rescued to full expression in the serum with the onset of recurrent HBV infection. In the experimental murine model, expression of HDAg was maintained for 6 weeks in human hepatocytes inoculated with HDV particles lacking HBV before being rescued to fully productive HDV infection by superinfection with HBV (54). In NTCP-transduced Hep G2 cells and dividing primary human hepatocytes, HDV replication was maintained after serial *in vitro* passaging despite blocking the extracellular spreading of HBsAg with Myrcludex (55), suggesting that HDV can survive liver regeneration and be amplified through human cell division both *in vitro* and *in vivo*. In further support to the hypothesis that HDV replication is more independent than currently appreciated, HBV integrants, which were naturally incorporated into the host DNA during infection, can produce HBsAg proteins competent for the assembly of HDV virions in the absence of ongoing HBV replication (42).

Origin of HDV

HDV has the smallest genome among animal viruses. Its genomic size of 1672–1697 nucleotides is slightly larger than plant viroids, which also have circular genomes and replicate by a rolling circle mechanism using the catalytic activity of ribozymes (56). Whereas viruses parasitize the host translational machinery, viroids are unique in parasitizing the transcription machinery of the host, as does HDV. HDV displays the dual behavior of depending on a preexisting host-encoded RNA polymerase as do viroids, but also on a virus-encoded protein like viruses. The similarity of HDV with a sequence in the human *CPEB3* gene encoding the cytoplasmic polyadenylation element binding protein 3 (57) and the molecular analogy between regions of the human 7SL RNA and the HDV sequence (58) led to the hypothesis that HDV could derive from a plant satellite RNA of smaller original size that acquired through recombination a cellular RNA encoding the HDAg and thereafter coevolved with the HBV (59). According to this hypothesis, HDV and viroids could be molecular fossils of a RNA world that presumably preceded our extant world based on DNA and proteins (60). HDV RNA may not be unique. Bioinformatic analyses, supported by biochemical evidence, indicate that hammerhead and HDV-like self-cleaving ribozymes are present ubiquitously and expressed throughout the tree of life (61, 62).

The positioning of HDV at the frontier of life relied on the circular conformation of the HDV RNA that was thought to be unknown to animal viruses and similar only to viroids. However, the recent recognition that many cellular RNAs are processed to form circular species resistant to host

nucleases has led to a speculative reappraisal of the origin of HDV. It has been proposed that some HBV RNAs may be processed to circular forms in hepatocytes infected with HBV, with the emergence and selection of circular species that can be replicated by host enzymes and assembled using HBV envelope proteins. The hypothesis is that the replicating RNA circle could undergo many nucleotide changes, rendering it unrecognizable relative to HBV sequences, and evolve to a genetically different infectious agent like HDV (63).

PATHOGENESIS

The histological pattern of hepatitis D is nonspecific, similar to the other types of viral hepatitis (64). A picture of small-droplet steatosis extensively involving liver cells that express HDAg (morula cells) was reported as typical of severe hepatitis D in natives of the Amazon Basin. This pattern has been rarely observed in developed countries.

Virus Factors

The pattern of disease varies in different epidemiological settings (65). In areas of heavily endemic infection, like northern South America, areas of Russia, Pakistan, and Mongolia, the course of hepatitis D has often been severe. A fulminant course was repeatedly observed in aboriginal communities and in military personnel stationed in the Amazon jungle (66). The high pathogenicity of HDV in hyperendemic areas may be related to an increase in virulence induced by the rapid circulation of the virus. Serial passage of HDV in chimpanzees and woodchucks results in a shortening of the incubation period and an increased severity of hepatitis D (67). The outcome is severe in intravenous drug abusers. These patients often have a concomitant HCV infection, which often accelerates the progression of the liver disease (68).

The genetic diversity of HDV may have a role in the different patterns of disease (Fig. 4) (69). Genotype III, which is unique to South America, appears to correlate with severe and fulminant hepatitis D among aborigines in the Amazon (70, 71). Genotype II isolated in Japan seems to cause only a mild HDV infection (72). Genotype I is prevalent in Europe and North America and segregates into two subgroups (IA and IB) (73). The HBV genotype may also modulate the infectivity of HDV (74) and high levels of HBV replication are associated with more severe liver damage (75).

The overload of viral genomes and genomic products in hepatocytes may be responsible for a cytolitic effect of HDV (76); however, expression of the s-HDAg or l-HDAg alone in transgenic mice has no cythopatic effect (Errore: sorgente del riferimento non trovata). Likewise no liver injury was observed after crossing HDAg transgenic mice with HBsAg transgenic mice. HDV RNA replication in HBV-free human hepatocytes maintained in the liver of chimeric mice did not induce cellular damage (77).

Host Factors and Immune Responses

The hypothesis that hepatitis D results from immune-mediated liver damage (78, 79) is supported by the type of inflammatory cells in the liver, variations in parameters of cellular immunity during HDV infections, and the finding of various autoantibodies in chronic hepatitis D. The latter include autoantibodies reactive against the microsomal membranes of the liver and kidney (LKM antibodies) (80), termed LKM3 to distinguish the virus-induced autoantibody from idiopathic LKM1 and from LKM2 elicited in hepatitis induced by tienilic acid. LKM3 is directed against an antigen of the UDP glucoronyl transferase 1 gene family (81).

Although a strong and persistent antibody response to the HDV is mounted after superinfection, this is not able to modulate the course of the infection. Cellular immunity may therefore be critical to the course of HDV infection. $CD4^+$/$CD8^+$ T-cell responses have been demonstrated in patients who cleared the HDV (82). The immune response to the virus involves the activation of HD antigen-specific helper T cells with the secretion of a variety of cytokines stimulating the further expansion of HDV-specific T cells. A higher frequency of $CD4^+$ cytotoxic T lymphocytes was found in HDV than in HBV or HCV disease and their presence was associated with elevated aspartate aminotransferase (AST) and diminished platelets (83). IFN-gamma is produced by activated HDV-specific Th1 and cytotoxic T cells, with the induction of class I and II major histocompatibility complex

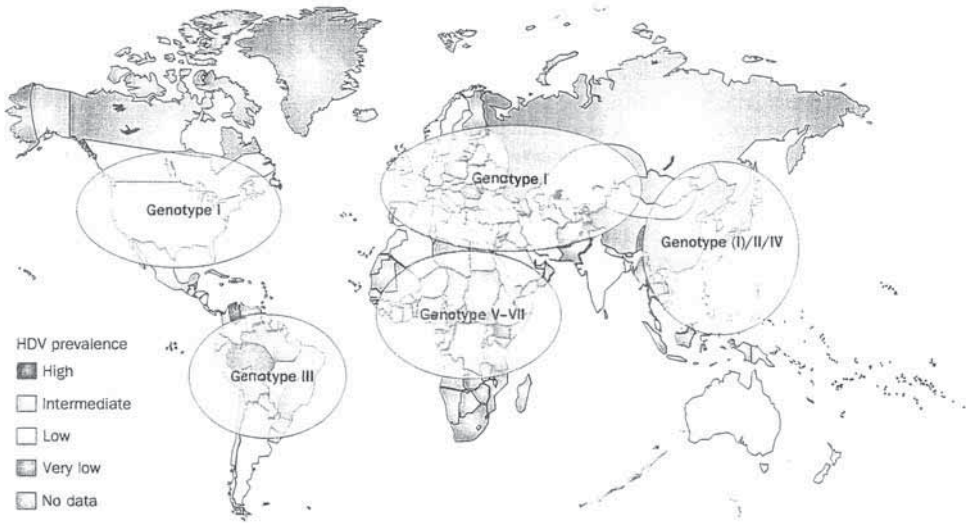

FIGURE 4 Global epidemiology of HDV infection according to viral genotype. (Reprinted with permission from reference [141].)

(MHC) proteins on hepatocytes and the secretion of protein 10 (CXCL 10), which further recruits natural killer (NK) cells (84). Cytotoxic CD8 lymphocytes specific for HLA-A2-restricted epitopes of the HDAg could be detected in mice vaccinated with HDV plasmid DNA.

In peripheral blood mononuclear cells stimulated with HDV/HBV peptides (85), responses to HBV-specific antigens were more frequent and robust than responses to HDAg peptides, suggesting that strong HBV-specific cellular immune responses occur in the majority of HBV/HDV co-infected patients. The enhancement of innate defense mechanisms is more prominent in HBV/HDV-infected than in HBV-monoinfected humanized mice (86), with a stronger induction of human IFN-stimulated genes and human specific cytokines, suggesting that the spontaneously elevated IFN levels may contribute directly to the inflammation and liver damage of HDV disease.

The liver damage of HDV may also result from interactions of its genome or gene products with defensive strategies of the host. The HDV counters endogenous IFN by directly inhibiting the activation of the IFN signaling pathways through the interference with Janus kinase/signal transducers and activators (JAK/STAT) (87). The large HDAg upregulates the *MxA* gene, which inhibits HBV replication, increasing the signaling of the tumor necrosis factor-induced nuclear factor-kappaB (NF-kappaB) (46), and sensitizes cells to inflammatory stimuli (88). The viral genome and its products appear to interact with the cell proteome (89, 90), presumably interfering with various steps regulating cellular metabolism, homeostasis, and growth.

EPIDEMIOLOGY

Distribution and Risk Groups

Infection with HDV is present worldwide. In the 1980s it was estimated that there were globally 15,000,000 carriers of HBsAg infected with HDV. Clinical studies have confirmed that hepatitis D was a major cause of cirrhosis and fulminant hepatitis worldwide (91). HDV is reemerging in the United States, where it appears to be uncommon in the general HBsAg population. Fifty percent of the chronically HBV-infected intravenous drug addicts studied in Baltimore, MD, in 2005–2006 had antibody to the HDAg (anti-HD) (92). In 2013, an 8% prevalence of anti-HD was found in 499 HBsAg carriers surveyed in Northern California (93); 69% were Caucasian non-Hispanic and 10% came from Asia or the Pacific Islands. The prevalence of HDV has diminished in Western Europe with the implementation of universal HBV vaccination that is depriving the HDV of its biological partner (94); however, despite steep declines in the general population in the 1990s, hepatitis D remains an issue in injection drug users, and is returning to Western Europe through immigration from areas where HDV remains endemic.

Incidence and Prevalence

The current prevalence of HDV infection remains underestimated for both lack of testing and inappropriate testing. Because of the perception that the decline of hepatitis D with the control of HBV meant that the disease was no longer a medical problem, testing for HDV has often been neglected in the last 20 years (95). In the United States, only 8.5% of 25,603 HBsAg-positive subjects observed from 1999 to 2013 were tested for anti-HD (96). In developing countries, increased resources are becoming available for HDV testing; however, testing is often performed in HBsAg carriers with no liver disease at low risk of HDV (94). Surveys for HDV must be disease-oriented, because carriers of HDV are more likely to be sick than healthy. To be comparable, prevalence rates should refer to a denominator of subjects with HBsAg liver disease.

The prevalence of HDV infection reported in the last decade in persons with chronic HBsAg hepatitis in different countries of Europe is shown in Table 2. Hepatitis D is rare in Australia (97, 98) and has significantly diminished in the general population of Taiwan, where the virus remains only in HBsAg carriers at risk (99). The prevalence of the infection is consistent in human immunodeficiency virus (HIV)/HBV subjects (100, 101). Endemicity is diminishing slowly in Romania and Turkey (94, 102). HDV is endemic in Pakistan (94, 103), in Iran (104), in Tajikistan (94), and is highly endemic in Mongolia (105, 106). Recently a 67% rate of anti-HD has been reported in the general HBsAg population of the country, amounting to an extrapolated figure of 135,936 HDV cases nationwide (Mongolia Viral Hepatitis Prevention Control Elimination Program, Poster 178. The Global Viral Hepatitis Summit. 15th International Symposium on Viral Hepatitis and Liver Disease, Berlin June 26–28, 2015, and Dr. Narambaatar Dashdory, Liver Center, Onom Foundation, personal communication).

Infection with HDV appears to be low in India (107, 108), Malaysia, Thailand, the Philippines, and Korea (94). It is endemic in North Vietnam; HDV RNA was found in 15.4% of HBsAg carriers in 43.3% of patients with acute

TABLE 2 Prevalence of anti-HD in chronic HBsAg hepatitis in Western Europe in the last decade

Study area	Positive/total (%)	Prevalence in immigrants	Reference
Hannover, 2009	252/2363 (9%)	80% from Turkey, Eastern Europe	68
Italy, 2014	87/1011 (8.4%)	40% from Eastern Europe	211
London, 2008	82/962 (8.5%)	85% from Eastern Europe, Asia, Africa	95
London, 2013	22/1048 (2.1%)		212
London, 2015:		60% from Africa, Turkey, Italy	213
Clinic-led testing	4/67 (6%)		
Reflex testing	158/3543 (4.5%)		
Switzerland, 2011	101/1699 (5.9%)		214
Belgium registry, 2013	44/800 (5.5%)	34% from Africa; 9% from Asia; 6.8% non-Caucasian	215
Athens, 2013	101/2137 (4.7%)	5% from the Balkans, Central Asia, Africa	216

Anti-HD = antibody to the HDAg; HBsAg = hepatitis B surface antigen.

HBsAg hepatitis (109) and seroprevalence rates of anti-HD were 30.2–29.4% in drug addicts in 2010–2011 (110). A 6.5% prevalence of immunoglobulin M (IgM) anti-HD was reported in a clinically mixed hospital HBsAg population collected in Guangdong, China, from 2005 to 2011 (111). HDV RNA was found in 37% of 54 patients with chronic hepatitis B in Kiribati, Western Pacific, but in no patients from Tonga, Fiji, and Vanuatu (112). Consistent rates of anti-HD were found in Gabon, Cameroon, and Nigeria (94). In 2014, antibody rates of 50% were reported in cirrhotic patients from the Central African Republic (113), of 11.3% in HBsAg liver disorders in Accra, Ghana (114), of 11.4% in pregnant HBsAg women in Northern Benin (115). The prevalence of HDV remains high in the western Brazilian Amazon (116).

Transmission

The parenteral route is the primary and most efficient for HDV transmission (64, 65). Carriers of the HBsAg are the major targets. Sufficient HDV was contained in 10^{-11} dilutions of a serum containing HBV/HDV to transmit HDV to a chimpanzee carrying HBsAg, yet the same inoculum transmitted HDV to HBV-naïve animals only up to 10^{-6} dilutions (117). In the former, HDV was rescued by the preexisting HBsAg of the recipient regardless of the original infectious titer of HBV in the inoculum; in the latter, transmission was limited by the source of HBsAg (i.e., by the infectious HBV titer of the inoculum), which was exhausted at 10^{-6} dilutions. Sexual transmission has been documented in prostitutes and sexual partners of HDV-infected carriers (118, 119). Cohabitation with an HDV carrier is a major risk for HDV transmission (94).

CLINICAL FEATURES

The clinical outcome of HDV infection is determined by the course of the concomitant HBV infection. In simultaneous co-infection (Fig. 5) in individuals previously not exposed to HBV, HDV can fully propagate only after HB viremia becomes established. In the great majority of cases, coinfections run an acute course with the clearance of HBV, and HDV cannot continue to replicate with the elimination of HBV; hepatitis D will then run a self-limited course (64, 120). Chronic HDV infection develops in less than 5% of the patients simultaneously coinfected by HBV and HDV.

In HBsAg carriers superinfected with HDV (Fig. 6), the preexisting HBsAg state promptly "rescues" HDV and favors the rapid establishment of infection. As the HBsAg carrier state is maintained over time, HDV superinfections most often become chronic, inducing new HDV disease in

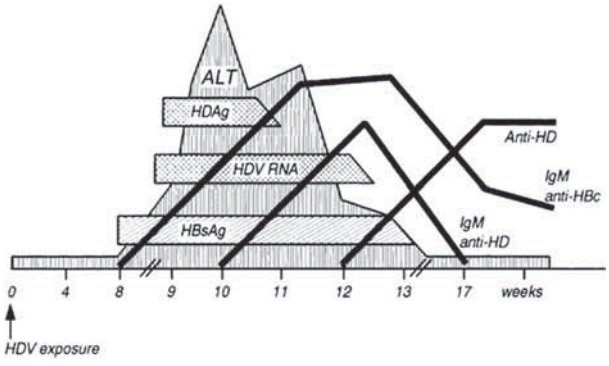

FIGURE 5 Serologic profile of HBV/HDV coinfection.

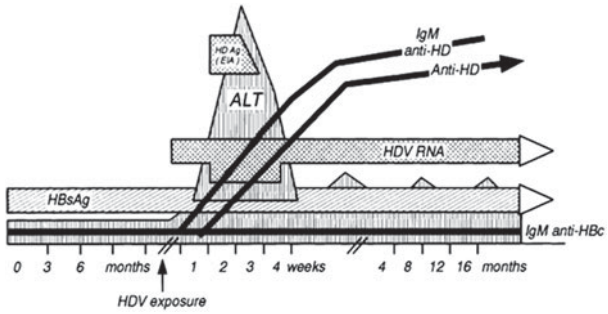

FIGURE 6 Serologic course of HDV superinfection evolving to chronicity.

healthy carriers of HBsAg or additional disease in carriers with previous HBV disease. HDV synthesis usually inhibits the replication of HBV. Patients with chronic hepatitis D have elevated alanine aminotransferase (ALT), lack hepatitis B e-antigen (HBeAg) and IgM anti-HBc, and have no or low titers of HBV DNA in serum. This atypical pattern of HBV in a patient with HBsAg-positive liver disease may indicate underlying HDV disease (121) and represents the "at-risk" HBV pattern indicating HDV testing (96). About 15% of patients with florid chronic hepatitis D raise LKM3 autoantibodies, which do not appear to have clinical significance or modify the response to IFN therapy.

Rarely, acute hepatitis D acquired by superinfection may run a self-limited course leading to the clearance of the preexisting HBsAg (122). In one study, the rate of HBsAg clearance over the years was increased in chronic hepatitis D compared to chronic HBV monoinfections (123). Superinfection with hepatitis D can resemble acute hepatitis B virus infection if the underlying HBsAg state of the patient was unrecognized, or may simulate reactivation of the underlying chronic hepatitis B virus infection (64, 121).

Active HDV infection without HBsAg rarely has been reported in immunocompetent patients; HDV RNA genotype I without the HBsAg was found in three Amerindians in Argentina who were negative for anti-HD but positive for anti-HBc (124), and HDV RNA without the HBsAg was found with anti-HD in a patient in Mongolia (125).

The clinical and histological features of chronic hepatitis D are not distinctive and are similar to HBV monoinfection. An occasional patient will have an enormous splenomegaly, unrelated to the degree of portal hypertension (Fig. 7). Increased accumulation of iron has been observed in the liver (126).

Clinical studies from all continents have shown that HDV infection aggravates the natural history of the underlying HBV infection (64, 121, 127). Hepatitis D is considered the most severe form of viral hepatitis in humans, accelerating the time of decompensation of liver function Table 3. The peak age of patients with cirrhosis associated with HDV is 10 years younger than those with cirrhosis due to HBV alone. The percentage and speed of progression to cirrhosis have varied in different series according to the characteristics of the HDV population studied (122,128–130). In a minor proportion of patients, the disease is less aggressive. In about 15% of patients followed in Italy, the course of the HDV liver disease progressed slowly (131).

The relative risk of developing cirrhosis in patients with HBV/HDV coinfection in Europe was estimated to be twice that of HBV alone (132). In about half of the patients with chronic hepatitis D, the disease is heralded by an overt acute

FIGURE 7 Massive splenomegaly in a patient with HDV cirrhosis (magnetic resonance of the abdomen).

hepatitis, which represents the time of HDV superinfection (133). In many patients, the disease is discovered incidentally, often at the stage of cirrhosis. Hepatitis D in children is acquired mainly by household contact and only rarely by vertical transmission. The course may be as severe as in adults (134). Due to the diminished circulation of HDV over the last two decades, the occurrence of newly recognized chronic hepatitis D has consistently diminished in Europe (132, 135), and the medical presentation has shifted from florid chronic hepatitis to residual advanced fibrotic disorders.

Triple HBV/HDV/HCV infection is frequently observed in injection drug users (136), with as much as 30% of such patients reported in central Europe (68). HDV was the dominant virus in European studies, inhibiting the expression of serum HCV RNA as well as of HBV DNA (137–139). HDV was also associated with the suppression of HBV and HCV in patients transplanted for triple infection (140). In a case report, chronic HCV was cleared upon superinfection with HDV (141). The HCV was the dominant virus in Taiwan (142). In a longitudinal study from Italy, viral dominance has changed over time with fluctuating HCV/HBV/HDV virologic profiles (143). Chronic hepatitis D appears to run an accelerated course in patients with HIV (144). In studies from Spain and Taiwan, HIV-infected patients coinfected with HDV were more likely to develop cirrhosis and had an increased risk of mortality compared with HDV patients without HIV (145, 146).

The contribution of HDV to the development of hepatocellular carcinoma (HCC) is controversial. Several oncogenic mechanisms have been proposed (147). In human Hep3B hepatocarcinoma-derived cell lines, full HDV replication was supported in the absence of HB virions (42). Also, HDV can infect the cells of hepadnavirus-induced HCC in woodchucks (148).

In a retrospective multinational European study, HDV infection increased the risk of HCC 3-fold compared to HBV monoinfection (133). After adjusting clinical and serological features, the estimated 5-year risk for HCC was 13% for anti-HDV positive patients versus 2–4% for patients with HBV infection alone. Forty percent of HDV-infected subjects in Greece with cirrhosis developed HCC within 12 years of follow-up (149). In a more recent study, the incidence of HCC in Italy was 2.8% per year (130). A comparison of HBV/HDV with chronic HBV in Swedish patients indicated that HDV was a strong risk for HCC (150); however, in an English study, the risk was not increased compared to HBV monoinfection (95).

The current risk for HCC with HDV/HBV compared to HBV monoinfection should be reconsidered according to the changing natural history of HBV; the latter can now be treated efficiently, and, therefore, deaths for HCC are increasing in HBV, whereas liver failure remains the major cause of death and reason for transplantation in HDV patients.

TABLE 3 Chronic hepatitis D features

Rapid progression to cirrhosis
Anti-HBe+; IgM anti-HBc–
HBV DNA low or absent
No specific histologic features
Occasionally splenomegaly +++

DIAGNOSIS

Antibody to HDV

Detection of anti-HD is the first step in the diagnosis of HDV infection (64, 151). Commercial radio- and enzyme-linked immunoassays are available for its detection. Antibody to the HDAg should be determined in all HBsAg carriers with liver disease, in particular in those at high risk for HDV infection, such as injection drug users. Active HDV infection is diagnosed by the finding of HDV RNA in blood or HDAg in the liver by immunohistochemistry. The sensitivity of immunohistology is limited because the antigen is not detectable in the liver biopsy samples of all patients with active HDV infections and has not been detected in 50% of the patients with advanced fibrosis (64).

Viral Antigen and RNA

Serum HDAg may be detectable only in the early phase of primary infection, before the development of the homologous antibody. As anti-HD is generated, HDAg becomes undetectable by immunologic assays, because it becomes masked in immune complexes (151).

Several in-house and commercial real-time PCR assays have been developed to quantify HDV viremia. The reverse transcription PCR (RT-PCR) technique has increased sensitivity allowing for qualitative and semiquantitative measurement of the viral genome (152). The assay is based on two steps—a first PCR with sensitivity of 1000 genomes/ml and a nested PCR with a detection limit of 10 genomes/ml (153). Assays use conserved primers from the carboxy-terminal segment of the HDAg-coding region, ensuring the highest degree of efficiency.

Detection of HDV RNA may help in the early diagnosis of acute infection during the seronegative period, in chronic infection of immunosuppressed individuals, in assessing the dynamics of HDV replication over time in coinfections with other hepatitis viruses, and in monitoring treatment response during antiviral therapy. With the current sensitive assays, serum HDV RNA is positive in 100% of HBsAg carriers with chronic HDV superinfection, correlating with

the intrahepatic expression of the HDAg. The level of HD viremia does not correlate with the stage of the liver disease (154, 155).

Results from different laboratories often are not comparable, and assays have not been standardized (156). In 2013, the first WHO International Standard for hepatitis D virus RNA for nucleic acid amplification techniques (NAT)-based assays was developed (157). Real-time HDV RNA quantification can be performed also in cryopreserved liver tissues specimens (45) and in formalin-fixed, paraffin-embedded tissues (158). Genotyping of HDV can be performed by restriction fragment length polymorphism analysis or by direct sequencing (73).

Coinfection Versus Superinfection

The serological patterns of coinfection and superinfection are distinct (Fig. 5 and Fig. 6). The HDV RNA, IgG anti-HD, and IgM anti-HD develop in primary HDV infection, either acquired simultaneously or upon a preexisting HBV infection. In self-limited HBV/HDV coinfections, markers of HBV replication (HBV DNA, hepatitis B e-antigen, IgM anti-core) are also present, whereas in superinfection of inactive HBsAg carriers or of those with HBeAg-negative chronic hepatitis B, these HBV measures are usually negative and anti-HBe is present at the onset of disease. A concomitant active HBV infection expressing the HBeAg is often seen in injection drug users. In acute coinfections, antibody markers are expressed only transiently, and HDV RNA may no longer be detectable at onset of disease (159). These measurements decrease rapidly and disappear after the clearance of the HBsAg (82, 160). In some patients, particularly in injection drug users, anti-HD can persist for years. All HDV markers persist in superinfection, with an increase to high titers of both IgG anti-HD and IgM anti-HD (161). The inflammation in the liver disease correlates with the titer of IgM anti-HD (162, 163). In patients with chronic infections, the IgM antibody is composed mainly of monomeric 7S IgM molecules (164), in contrast to the predominance of 19S pentameric molecules in acute infections. HDV RNA remains detectable with titers fluctuating over time.

PREVENTION

Vaccination against the HBV protects from HDV infection; its implementation has much diminished the incidence of HDV infections by reducing the reservoir of HBsAg carriers susceptible to HDV. There is no immune prophylaxis to protect the HBsAg carrier from HDV superinfection. Antibodies to the HDAg do not neutralize the HBsAg-coated virion and are not protective. Therefore, virus-specific T-cell immunity appears to be required for protection (165). DNA vaccines expressing the small and large HDAgs have elicited cellular immune responses in mice (166), but vaccination with protein or DNA vaccine in woodchucks failed to protect the animals (167). Protection against coinfection with HDV and woodchuck hepatitis virus was achieved using DNA priming and adenoviral boost regimen (168), but the T-cell response in the preclinical woodchuck model by current immunization procedures seems insufficient to prevent the spread of HDV in chronic HBV carriers.

THERAPY

Therapeutic strategies aimed at inhibiting functions of HDV for hepatitis D are constrained because the virus expresses no enzymatic target and relies on the replicative machinery of the hepatocyte. The current therapy of the disease remains empirical; it is based on IFN-alpha, introduced in clinical practice 30 years ago (169–171). *In vitro*, IFN has no effect on HDV RNA (172); the virus may directly inhibit IFN-alpha signaling (87). IFN appears to delay the entry of HDV into hepatocytes (173).

Standard IFN for 1 year at dosages of 3–6 million units (MU) three times weekly induced disease remission (normal ALT) in ∽20–25% of patients with chronic hepatitis D (169–171). Rates of HDV RNA clearance were lower. In most studies, results were worse in cirrhotics. The degree of response was proportional to the dose of IFN. Patients given 5 MU daily or 9 MU three times per week responded better than patients given lower dosages (174). IgM anti-HD declines and then disappears in responders (164, 175).

Long-acting peg-IFN-alpha has marginally increased efficacy (176–180). In four studies, a virologic response was observed in 18–43% of the patients (Fig. 8). In a study by the Hep-Net International Delta Hepatitis Intervention Trial (HIDIT) (181), 90 patients were randomly assigned to receive either 180 μg of peg-IFN alpha-2a weekly plus 10 mg of adefovir (31 patients), 180 μg/kg peg-IFN plus placebo (29 patients), or adefovir alone (30 patients). By week 48 of therapy, the reduction of HDV RNA was higher and similar in the two peg-IFN groups compared with adefovir alone. At 6 months posttherapy, HDV RNA was negative in 28% of patients given peg-IFN compared with only 8% of the patients given adefovir alone.

In the HDIT 2 (182) study of peg-IFN monotherapy versus peg-IFN plus tenofovir for 96 weeks, the combination treatment had similar efficacy and safety profiles compared to peg-IFN therapy alone. Long-term IFN-treatment of HDV patients caused a selective loss of terminally differentiated NK cells with an enrichment in the immature NK cell subset and treatment was associated with marked functional impairment of NK cells and reduced signaling via STAT4 (183). No difference in response to peg-IFN was observed between treatment-naïve and treatment-experienced patients; the individual interleukin 28B polymorphism was not predictive of response (184, 185). Increasing the dosage of IFN, prolonging therapy to 24 months, or adding an antiviral against HBV or ribavirin to peg-IFN conferred no advantage (186).

During IFN therapy, the decline of HDV RNA is biphasic, with a first rapid phase of about 1 month duration

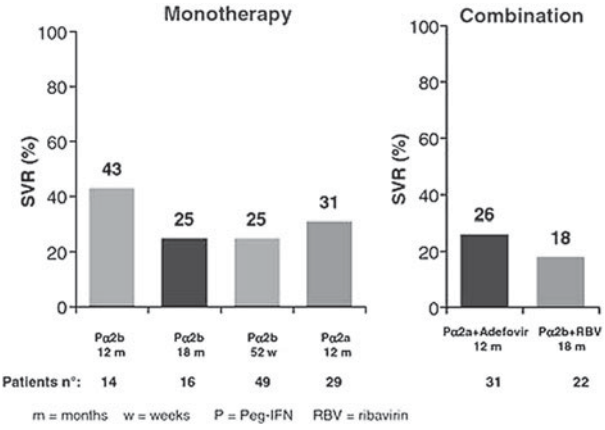

FIGURE 8 Chronic hepatitis D treated with Peg-IFN monotherapy or in combination with antivirals.

followed by a second slower phase (187). The decline of HDV RNA during early treatment time points does not seem predictive of a sustained viral response (SVR) (188). A negative HDV RNA test at 6 months of therapy is a better predictor of a SVR. Treatment is indicated in patients with active compensated HDV disease. In patients with advanced cirrhotic disease, the expected benefits of therapy should be balanced against the adverse effects of peg-IFN and the lower rate of response.

Of note, current assays for the measurement of HDV RNA have a detection limit of no lower than 10 viral genomes/ml, which is much more than the natural infectivity threshold of HDV for the HBsAg carrier (117); thus, clearance of HDV RNA determined with these assays does not ensure elimination of infectious virus. Therefore, in patients who achieve an SVR but remain HBsAg-positive, residual undetectable HDV may still be present at low titers and able to reactivate hepatitis D after apparently successful therapy (189, 190). In a 4.5-year median follow-up in the HIDIT trials, 56% of 16 patients with undetectable HDV RNA 6 months posttherapy returned positive for HDV RNA at least once during the posttherapy follow up and 7 retested positive at the last visit (191). Virologic relapses were associated with ALT increases in at least 4 subjects.

The elimination of the HBsAg is the most reliable endpoint of therapy (192–194), but this goal is seldom attained. In the long-term virologic responders of the HIDIT study, serum HBsAg had decreased at week 48 of therapy by a mean 1.6 log IU/ml, whereas HBsAg levels showed an increase of 1.0 log 10/ml in individuals with late relapses.

To push for eradication of HDV, long-term therapy with IFN over several years could be proposed in patients who exhibit a significant decline of HBsAg during initial therapy (189). The current recommended schedule for the treatment of chronic HDV disease is peg-IFN-alpha given weekly for 48 weeks; however, the optimal duration of treatment has not been established. Longer durations of treatment may be appropriate on an individual basis in patients in clinical remission who remain viremic to maintain control of disease despite the persistence of HDV (195).

Antivirals against the HBV that inhibit HBV DNA synthesis but leave the HBsAg unaffected have no role in hepatitis D (181, 189). Attempts to cure the disease by depriving the HDV of the partner HBV using lamivudine, adefovir, entecavir (196), or tenofovir had no effect. In only one study of HDV-HIV did coinfected patients given tenofovir for a median of 6 years (197) therapy achieve a distinct reduction of HDV RNA in 13 of 16 patients. An antiviral against the HBV is advisable in patients with significant HBV DNA serum levels, although control of HBV does not modify the natural course of the HDV liver disease.

New therapeutic strategies are being explored that aim at interfering with steps of the HDV cycle different from replication of the virus. Two approaches in clinical development are the interference with the assembly of the HD virion and the inhibition of HDV entry into or exit from hepatocytes by the blocking of the receptors for the HBsAg or the synthesis of subviral HBsAg particles. A model of the first strategy is the disruption of prenylation of the l-HDAg. In hepatocyte cell culture, prenylation (farnesylation) of the l-HDAg was inhibited by the prenylation inhibitor BZA5B (198), and the prenylation inhibitors FTI-277 and FTI-2153 were effective at clearing HD viremia in a mouse model of HDV infection (199). In a small blinded, randomized, placebo-controlled study (200) of patients with chronic hepatitis D given the farnesyl transferase inhibitor lonafarnib at 200 mg (100 mg twice daily) or 400 mg (200 mg twice daily), the mean log HDV RNA declined 0.73 log IU/ml from baseline in patients given 200 mg and 1.54 log IU/ml in patients given 400 mg by day 28. The level of serum HBsAg remained unchanged. No HDV mutations associated with lonafarnib nonresponse were detected by population-based sequencing for the l-HDAg. Adverse events were frequent, with 50% and 33% of the patients given the 200-mg dose experiencing diarrhea and nausea, respectively, and all patients given the 400-mg dose experiencing nausea, diarrhea, abdominal bloating, and weight loss greater than 2 kg. Ritonavir boosting can further increase the antiviral effect of lonafarnib (201).

A variety of drugs can inhibit the NTCP receptor for HBsAg (202). Mircludex (203), a myristoylated 47-amino-acid fragment of the pre-S1 region of the large HBV envelope protein (pre s-1 peptide), which binds to NTCP and acts as a competitive inhibitor of HBsAg entry, induced a >1 log decline of HDV RNA in serum and the temporary control of the development of HDV infection in superinfected HBV mice (204).

In a preliminary study, the nucleic acid polymer REP-2139 (205), given once weekly by a 2-hour intravenous infusion at a 500-mg dose, first as monotherapy and then with the addition of peg-IFN at week 16, induced a 4–5 log reduction of serum HBsAg and a 5–8 log reduction of HDV RNA in 4 of 7 treated patients. Anti-HBs became detectable in 6 patients.

LIVER TRANSPLANTATION

Prevention of the recurrence of HBV by using passive immunoprophylaxis with standard HB immunoglobulin against the HBV is satisfactory for the prevention of HDV reinfection (206). The reinfection rate of HDV dropped to 9–12% (207), with a survival rate of 98% throughout a 7- to 10-year follow up. Prophylaxis with lamivudine pretransplantation and with lamivudine plus HB immunoglobulin posttransplantation has further diminished the residual rate of reinfection. Of note, HBsAg-positive grafts must not be given to recipients with HDV coinfection. In two such cases, hepatitis D rapidly recurred in the transplanted patient (208, 209). Likewise the choice of maintaining prophylaxis with antivirals without further HBIg may be debatable in HDV transplants; the reemergence of HBsAg, which may be innocent in the ordinary HBV transplant protected by antivirals (210), might by itself rescue HDV to pathogenic expression if this virus has remained latent in the transplanted liver.

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Prion Diseases

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Prion diseases are infectious and fatal neurodegenerative disorders of humans and animals caused by the accumulation of a misfolded and aggregated form of the cellular prion protein (1, 2). The term "prion" was coined by Stanley Prusiner and is derived from the words "proteinaceous infectious particle" (2). Prions are misfolded forms of a normal protein called the "prion protein" and by definition are infectious. In most prion diseases, prions are abundant in the brain and spinal cord and can spread between patients iatrogenically, for example, through neurosurgical procedures or grafts of prion-contaminated dura mater (3). The classic neuropathologic lesion neuropatholog in the brain of a prion-infected patient is spongiform degeneration with neuronal loss, activated astrocytes and microglia, and a notable lack of peripheral inflammatory cells (4).

HISTORICAL BACKGROUND

The prototypic prion disease, scrapie, was described in 1750 as a fatal disease of sheep and goats and was demonstrated to be infectious in the 1930s (5). Human spongiform encephalopathy cases were initially reported in the early 1920s (6, 7), but were not shown to be transmissible until several decades later (8, 9). In 1957, Carleton Gadjusek reported that an unusual epidemic of a neurologic disease known as "kuru," meaning to shiver, had struck the Fore people of Papua New Guinea (10). In a letter to *Lancet*, veterinary pathologist William Hadlow noted that the brain lesions in kuru patients resembled those of scrapie-affected sheep and suggested these diseases may share a similar infectious etiology (11). Experimental injection of the kuru-affected brain into macaques was subsequently shown to cause a spongiform encephalopathy and led to the realization that kuru was spreading among the Fore as an infectious disease from endocannibalistic practices (9). For these findings, Gadjusek was awarded the Nobel Prize in Medicine or Physiology in 1976 for "*discoveries concerning new mechanisms for the origin and dissemination of infectious diseases*." The pioneering discovery of kuru as an infectious disease set the stage for the recognition of other rapidly progressive neurodegenerative diseases as part of a family of prion diseases, including Creutzfeldt-Jakob disease (CJD) in humans, bovine spongiform encephalopathy (BSE) or "mad cow" disease in cattle, scrapie in sheep and goats, and chronic wasting disease (CWD) of deer and elk.

The history of the nomenclature for CJD is complicated. Alfons Jakob published four papers in 1921 and 1923 describing five uncommon cases of rapidly progressive dementia, stating that his cases were quite similar to a previously published case reported by his professor, Hans Creutzfeldt, in 1920. This disease was referred to as Jakob's or Jakob–Creutzfeldt disease for many decades until a prominent researcher in the field, Clarence J. Gibbs, began using the term Creutzfeldt–Jakob disease, as its acronym, CJD, was closer to his own initials (12). Interestingly, the cases Jakob described were in fact very different than Creutzfeldt's case, in that only two of Jakob's five cases had pathological evidence of the disease that we now consider to be prion disease, whereas Creutzfeldt's case did not have prion disease (13). Thus, the name for human clinical prion disease should be Jakob's disease or possibly Jakob–Creutzfeldt disease. However to avoid confusion, in this chapter we use the more commonly used term Creutzfeldt–Jakob disease (CJD).

More recently, prion diseases were recognized for their potential to cause large-scale epidemics and spread as zoonotic infections. BSE was first described in 1986 (14), and by June 1996, more than 180,000 confirmed cases of BSE in cattle had been reported within Great Britain (15). By 1995, a new human variant of Creutzfeldt-Jakob disease (vCJD) appeared in the UK, likely from human exposure to BSE-contaminated beef (16, 17), and has since led to more than 200 deaths from vCJD. During the time of the BSE epidemic, there were many key research breakthroughs in the prion field, including the identification of the causative agent as an infectious aggregated protein (2), which earned Stanley Prusiner the Nobel Prize for Medicine or Physiology in 1997.

In 2004, the first major breakthrough in defining prions as infectious proteins occurred when Giuseppe Legname and colleagues demonstrated that recombinant mouse PrP fibrils (from amino acids 89-230) were infectious in transgenic mice expressing mouse PrP fragment 89-231 (18). In 2008, Surachai Supattapone and colleagues showed that the minimum components for generating an infectious prion were PrP^{C}, co-purified lipids, and a polyanion (19). By 2010, another major breakthrough from Jiyan Ma and colleagues showed that recombinant PrP in the presence of lipids and RNA forms prions were highly infectious to wild-type mice

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(20). This landmark experiment demonstrated for the first time that recombinant prion protein containing co-factors was sufficient to form an infectious prion as postulated by Dr. Prusiner and was key to excluding viral particles or other infectious agents as necessary for prion infectivity.

PRIONS

Classification

The classification of prions differs from that of viruses because prions are infectious proteins. Thus, there are no prion families or genera. Instead, prions are classified by their host species, clinical disease, pathologic lesions in the brain, and the molecular and biochemical properties of the aggregated prion protein. The primary amino acid sequence of the prion aggregate is determined by the host cellular prion protein, PrP^{C}, encoded by the prion gene on chromosome 20 in humans (21). No specific nucleic acids are required, and neither ionizing or UV radiation (22–24), treatment with nucleases (2), nor formalin inactivates prion infectivity (25).

The human prion diseases include sporadic, genetic, and acquired forms of disease, the most common form being sporadic CJD (sCJD). The cause of sCJD is not known but has been hypothesized to be due to a somatic mutation arising in the gene encoding the prion protein gene, *PRNP* (26) or from the spontaneous conformational conversion of PrP^{C} (in which "C" stand for the normal or cellular form of the protein) into the aggregated, pathogenic form, PrP^{Sc} (in which "Sc" stands for scrapie, the prion disease of sheep and goats). Genetic prion diseases have been historically classified primarily by clinical symptoms and neuropathological features as familial CJD (fCJD), Gerstmann-Sträussler-Scheinker (GSS) disease, or fatal familial insomnia (FFI). However, this classification was prior to the discovery of *PRNP*. The mutations in *PRNP* consist of missense, insertion, and deletion mutations and are autosomal dominant and typically highly penetrant, with disease onset in the fifth or sixth decade (27). Acquired prion diseases have been transmitted between individuals (kuru and iatrogenic CJD or iCJD) or in some cases, from cattle to humans (vCJD) (16, 28).

Prion diseases of animals are thought to be largely acquired by exposure to infectious prions, however, some may arise sporadically, such as atypical scrapie in sheep and atypical BSE in cattle (29–31). Classical scrapie affects sheep and goats nearly worldwide, and the name "scrapie" refers to the clinical disease, because prion-infected sheep develop pruritus and typically scrape against fences (32). Classical BSE first appeared in cattle, possibly a result of transmission of a case of sheep scrapie to cattle or from a sporadic TSE in aged cattle. BSE spread to zoo bovids, primates, including humans, and felids, likely through BSE-contaminated food products (33–39). Chronic wasting disease (CWD) was first discovered in Colorado deer in 1967 (40) and currently affects deer, elk, and moose (family Cervidae) in more than 23 U.S. states and two Canadian provinces, as well as ranch-raised elk in South Korea (41). Transmissible mink encephalopathy (TME) has been identified in farmed mink in the United States, Canada, Russia, Finland, and East Germany and was thought to be due to dietary exposure to a prion-infected animal, although the origin of the epidemic remains unclear (42).

Phenotypic Variation

A remarkable observation in prion biology that has intrigued scientists for decades is the prion strain phenomenon. The variation in clinical symptoms and histopathology among patients expressing the same *PRNP* sequence challenged Prusiner's protein-only hypothesis; how could this phenotypic variability be explained in the absence of a genome from an infectious agent? Extensive experimental data now support that the variation arises due to multiple conformations of PrP^{Sc} (43–47). In rodent models, distinct prion strains can be propagated in animals expressing the same PrP amino acid sequence yet differing in the clinical and pathological phenotype. Classical methods to identify distinct prion strains utilize inbred mice to demonstrate differences in the disease incubation period and the regions targeted in the brain as measured by the degree of spongiform change, gliosis, and PrP^{Sc} aggregates in select brain regions (48–50). Serial passage of a single prion strain in mice typically "breeds true," meaning that strains show a remarkable conservation in the incubation periods and lesion distribution. Biochemical differences, such as the stability in chaotropes or heat, can aid in distinguishing strains and are indicative of conformational differences in PrP^{Sc} (43–47). Extensive biochemical studies of PrP^{Sc} have collectively shown that multiple different arrangements are likely to explain the observed differences in disease properties (51, 52), although the precise PrP^{Sc} conformation(s) and how they differ are not yet clear.

Composition

PrP^{C} contains 253 amino acids, of which peptides 1–22 and 232–253 are cleaved during processing. The mature, processed PrP^{C} is composed of 210 amino acids arranged with a disordered amino terminal domain and a well-ordered, C-terminal globular domain containing three α-helices and a short anti-parallel β-sheet (53, 54) (approximately 42% α-helical and 3% β-sheet structure (55)) (Fig. 1A). Post-translational modifications include two variably occupied N-linked glycan sites in the C-terminal globular domain and a glycophosphatidylinositol anchor (GPI) that attaches PrP^{C} to the cell membrane (M_r 33,000–35,000) (56–58), which is also present in PrP^{Sc} (57).

Conversion of PrP^{C} to PrP^{Sc} involves a massive structural rearrangement of the primarily α-helical protein into a β-sheet-rich structure (approximately 47% β-sheet) (59) (Fig. 1B). PrP^{Sc} templates the misfolding of the host PrP^{C}; thus, the prions that accumulate in the host will have the PrP amino acid sequence of the host (Fig. 1C). The detailed mechanism by which PrP^{C} is converted into PrP^{Sc} remains unknown. One hypothesis is that short segments of PrP^{Sc} interact with PrP^{C} in a "steric zipper," in which complementary amino acid side chains from two β-sheets tightly interdigitate and stabilize growing fibrils (60, 61).

Highly conserved among mammals, PrP^{C} is expressed nearly ubiquitously by most cells, and at notably high levels in neurons. Multiple functions have been assigned to PrP^{C}, including signaling important for myelin maintenance in the central and peripheral nervous system (62), as well as metal binding, particularly copper (63, 64), cell signaling, potentially through fyn activation (65, 66), and neuroprotective signaling (67–69). Aged PrP knockout mice show demyelination in peripheral nerves (62, 70) and alterations in circadian activity and sleep regulation (71, 72).

Although both crystallography (73) and nuclear magnetic resonance (NMR) spectroscopy (53) have yielded detailed insights into the structure of PrP^{C} at the atomic level, the insoluble PrP^{Sc} aggregates have presented a challenge for structural determination. Studies using x-ray crystallography of prion fibrils purified from brain indicate PrP is

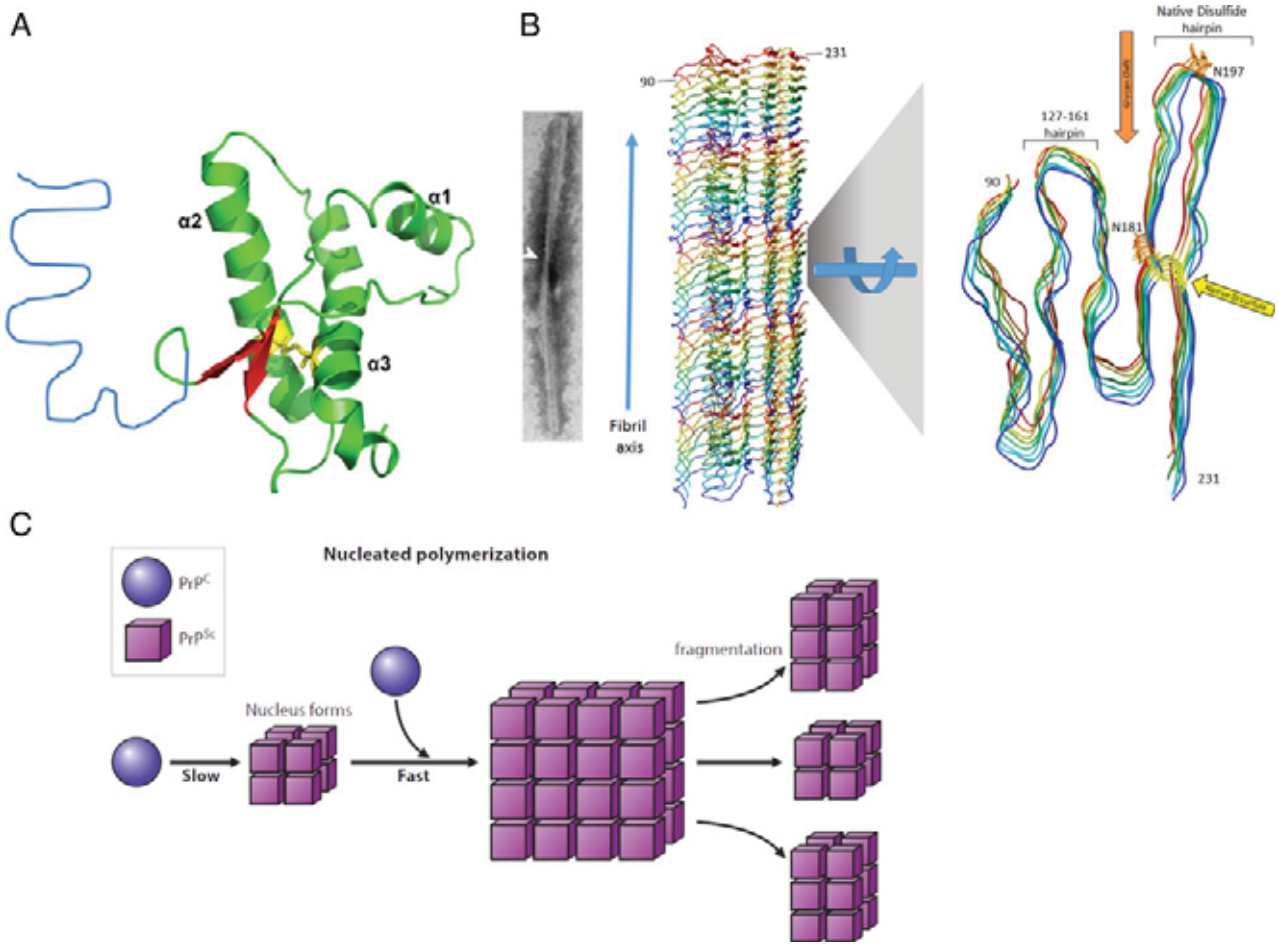

FIGURE 1 (A) The structure of mouse PrP^C solved by NMR spectroscopy (53) shows an unstructured amino terminal domain (blue) and a well-ordered C-terminal domain with three α-helices (green) and an anti-parallel β-sheet (red). A disulfide bond links α2 to α3 (yellow). (B) A long straight fibril from PrP^{Sc} derived from a prion-infected brain shows a single twist (arrowhead) (292). A model of the fibril composed of multimeric PrP^{Sc} is shown that accounts for available biophysical measurements (75). Note that all α-helices have been converted to β-sheets, and PrP molecules are stacked. Each PrP molecule is represented by a different color and molecules are aligned parallel and in-register. The locations of the glycans are shown in orange. (C) Model for the conformational conversion of PrP^C into PrP^{Sc}. The "seeding" or nucleated polymerization model proposes that PrP^C forms a highly ordered nucleus, which requires overcoming a high-energy barrier. Further monomeric PrP^C is recruited into the growing PrP^{Sc} aggregate. Fragmentation of PrP^{Sc} aggregates increases the number of nuclei, which each recruit PrP^C monomers, resulting in amplification of the prion aggregates. (Modified from references [293] and [75]).

arranged in a cross-β-sheet structure similar to other amyloids (74). Recent studies suggest a model in which PrP^{Sc} is arranged in a super-pleated parallel sheet (Fig. 1B) with the glycans tucked within a glycan cleft (75). A second model of PrP^{Sc} also shows a loss of all α-helical structure and instead shows a β-sheet enriched, highly ordered arrangement of multimers (76).

Notable characteristics of prions include (1) development of very high titers of infectivity in the brains of their hosts—laboratory strains passaged in hamsters can reach titers of $>10^8$ ID_{50} per ml of brain homogenate (ID_{50} is the dose that will infect 50% of the experimental group) (77); (2) a particle size as small as 30 nm (78); (3) highly infectious prion particles composed of only 14–28 PrP molecules (79); (4) high resistance to ultraviolet and ionizing radiation, having a very small radiation target size (23, 24); (5) polymerization after proteinase-K digestion to form helically wound amyloid fibrils 4 to10 nm in diameter visible by electron microscopy (80); and (6) no detectable immune responses evoked in their host (81, 82).

Biology

Replication strategy

During an exposure to infectious prions, the incoming infectious PrP^{Sc} templates and catalyzes the conformational conversion of host PrP^C. Thus, PrP knockout mice completely resist prion infection, and reintroduction of the PRNP gene by transgenesis restores prion disease susceptibility in mice (69,83–85). Conversion of PrP^C to PrP^{Sc} is promoted by cofactors, including phosphoethanolamine, RNA, or glycosaminoglycans, and the prion particles frequently contain lipids and glycosaminoglycans, which may be necessary for conversion to the infectious form (19, 20, 86).

In yeast, fragmentation of Sup35 prion aggregates by chaperones enhances and accelerates conversion, likely by

generating additional aggregate ends, each of which templates the conversion of additional monomers (87). Similarly, in mammalian prions, sonication markedly enhances prion conversion and amplification *in vitro* by a technique known as protein misfolding cyclic amplification (PMCA) (88).

Host range and tropism

Although prions typically spread most efficiently to individuals within a species, prions can also spread between species. In general, prions transmit most efficiently between individuals having similar PrP sequences (89, 90); for example, sheep scrapie prions can transmit the infection to elk (91). That said, certain host species are surprisingly susceptible to prions from evolutionarily distant species. As a potential rodent wildlife reservoir of prions, bank voles quite unexpectedly have been found to be highly susceptible to prions from many species (92, 93). Thus bank voles have been referred to as a "universal acceptor" for prions. Transgenic mice expressing bank vole PrP are also susceptible to prions from many species, indicating that the bank vole PrP sequence, and not any vole specific co-factors, underlies their high susceptibility (94). Several vole species in North America (prairie, meadow, and red-backed vole) are also highly susceptible to CWD prions (95, 96).

The use of transgenic mice expressing chimeric PrP^{C} molecules has led to two major principles that govern prion transmission into a new species: (1) the incoming (exogenous) PrP^{Sc} interacts preferentially with a host PrP^{C} having a similar primary amino acid sequence, particularly at certain residue positions (89, 90) and (2) the PrP^{Sc} conformation impacts species barriers. Species susceptibility to prions can be manipulated by exchanging a few amino acid substitutions in the host PrP^{C} sequence. For example, transgenic mice expressing human PrP^{C} resist infection with elk CWD, but transgenic mice expressing human PrP with 4 elk amino acid substitutions at a key segment for conversion are highly susceptible to CWD prion infection (97).

Intriguingly, even a single amino acid difference in a key position can alter prion susceptibility. Humans have a common coding polymorphism at *PRNP* codon 129, encoding either methionine or valine, which in the general Caucasian population consists of approximately 37% Met/Met, 51% Met/Val, and 12% Val/Val CJD (98). All but one of the clinical vCJD cases to date have been in individuals expressing PrP-129MM, indicating that BSE prions more efficiently convert human PrP-129MM (99, 100). A few cases of asymptomatic individuals heterozygous for PrP-129 (MV) have been found to have prions in their lymphoid tissue (101, 102). Whether there will be a second wave of vCJD cases in individuals expressing human PrP-129VV or -129-MV is unknown.

Growth in cell culture

Prions can be propagated in a variety of PrP^{C} expressing cultured cells, commonly in N2a neuroblastoma cells, and interestingly, replication can lead to high titers of infectious prions without causing cell death (103, 104). Prion-infected N2a cells have been used to screen drug libraries for antiprion therapeutics, (103). Prions can also be propagated in neurospheres (104) and primary neuronal cultures (105).

EPIDEMIOLOGY

Human prion disease has a reported incidence worldwide of approximately 1 to 1.5 cases per million individuals per year based on mortality data (106), although the prevalence is higher in some countries, particularly those engaged in more active surveillance (107). Nevertheless the number of cases per year is fairly constant within and between countries and ranges from 0.41 cases per million in 1994 in Spain to 2.63 cases per million in 2002 in Switzerland (108), with an equal incidence in men and women (109). This incidence of prion disease is consistent even in countries free of classical sheep scrapie, supporting the evidence that scrapie is not transmitted directly to humans. sCJD is the most common human prion disease and comprises approximately 85% of human prion disease cases. Homozygosity at codon 129 (MM or VV) is a risk factor for the development of sCJD or acquired CJD (98, 110). Although the incidence of prion disease is 1 to 1.5 cases per million, a person's lifetime risk of dying from CJD is much higher for several reasons. Incidence is across the entire age spectrum, from infants to the very elderly, whereas the median age of onset for CJD is a unimodal peak around age 67 (111), an age that most people in the developing world would reach. Taking all deaths from CJD over all deaths and considering that most persons will live past their mid-60s, the lifetime risk of dying from CJD is estimated to be about 1 in 30,000.

sCJD is a heterogeneous disease entity and includes a wide variety of clinical and histopathological phenotypes. The codon 129 polymorphism is partly responsible for this phenotypic diversity and is a major genetic modifier; thus human sCJD prions can be classified based on the codon 129 genotype as well as the conformational properties of PrP^{Sc}. For example, the electrophoretic mobility of PrP^{Sc} depends on the size of the proteinase K (PK)-resistant PrP^{Sc} core size. Type 1 shows a PK-digested, unglycosylated fragment size of 21 kilodalton (kDa), whereas type 2 shows a fragment of 19 kDa (112). The codon 129 polymorphism shows some influence on the PrP^{Sc} type that develops, as ~95% of 129MM sCJD patients have the type 1 pattern, whereas ~86% of 129MV or 129VV have the type 2 pattern (111, 113, 114). Individual patients may also have both type 1 and type 2 patterns in the same or different brain regions (111,115–117).

Numerous case-control studies have been conducted to identify risk factors for sCJD and have typically enrolled small numbers of cases and controls with different methods and contradictory results. Collectively, studies have not thus far provided any conclusive evidence of a higher risk for sCJD in relation to occupation, animal contact, diet, or history of blood transfusion (118–121). One European Union collaborative study on CJD that examined medical and associated risk factors from 326 patients with sporadic CJD compared with 326 community controls found increased risk from surgery as well as from ear piercing and psychiatrist visits (121). An impressive study from Denmark and Sweden, two Scandinavian countries with national health systems in which actual records of surgeries were used (thus avoiding recall bias) found an increased risk for sCJD from various surgical procedures (121, 122).

Genetic prion diseases encompass approximately 15% of prion diseases and are caused by more than 30 missense point mutations and octapeptide repeat insertions (1, 2, and 4–9 additional repeats) in the *PRNP* open reading frame, inherited in an autosomal dominant pattern (123, 124) (Fig. 2). The most common human *PRNP* mutation worldwide is CJD-E200K-129M, which has a penetrance of 60% to 90% and shows an average onset age of 58 years (125, 126). Human familial prions were shown to be transmissible (127, 128), and transgenic mouse studies later replicated properties of familial prions in generating familial prion diseases transmissible to mice (129–131).

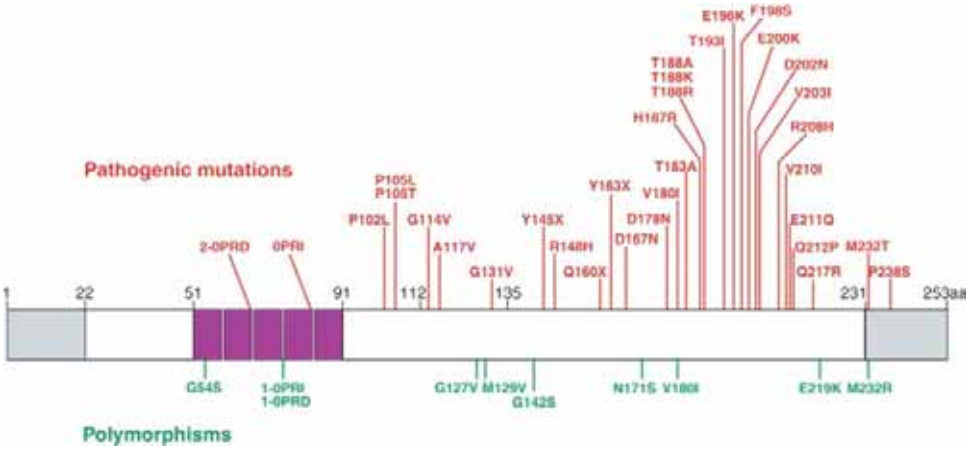

FIGURE 2 Schematic representation of human PrP showing the known mutations and polymorphisms. The cleaved signal sequences are shown in dark gray and the octapeptide repeat region in purple. Shown are the pathogenic mutations (red) and the nonpathogenic variants (green). OPRD: octapeptide repeat deletion; OPRI: octapeptide repeat insertion. (Modified from reference [294]).

Iatrogenic prion transmission was first recognized in 1974 with prion transmission from a corneal graft. Additional iatrogenic cases followed in individuals exposed to prion-contaminated dura mater grafts, growth hormone derived from human pituitaries, neurosurgical instruments, and EEG electrodes. The sources of the infectious prions in these cases were either directly from the brain parenchyma or from tissues adjacent to the brain, such as meninges, that also contain high levels of prion infectivity. The highest number of iatrogenic transmissions have occurred from growth hormone derived from human pituitaries (162 cases) followed by dura mater grafts (136 cases) (132). Latency period from prion exposure to the development of clinical disease in iatrogenic prion transmission cases have ranged from 1.5 to 16 years postexposure (132, 133).

The risk of sCJD transmission through blood transfusion was evaluated in a recent study of 29 sCJD blood donors and 211 transfusion recipients. None of the deceased or living recipients have developed sCJD, consistent with the negative data in other studies (134), suggesting that transfusion transmission of sCJD is unlikely (135). In contrast to the sCJD group, there have been three cases of transfusion-transmitted vCJD linked to vCJD-infected blood donors out of a total cohort of 67 (4.5%), 34 of which were successfully traced recipients (50.7%) (136–138). A single additional case showed PrP^{Sc} in the spleen postmortem (101). In a sheep model, all clinically relevant blood components transmitted prion infection to recipients in a single transfusion event (139).

Variant CJD is hypothesized to be caused by dietary exposure to BSE; there were 229 cases by 2015, 177 of which were from the United Kingdom. The vCJD epidemic showed an increase in cases from 1995, peaked in the year 2000, and has since dropped steadily, with no cases reported in 2015. All pathologically confirmed vCJD cases to date have occurred in patients who were homozygous for methionine at codon 129 of *PRNP*, compared to 37% of the general Caucasian population (98). A recent retrospective study of archived appendices in the United Kingdom, however, revealed 16 out of 32,441 appendices harbored PrP^{Sc}, 8 of which were 129MV or VV, suggesting that 1:2000 people in the UK could have a long latency period and may be subclinical carriers of infectious prions (102). Furthermore, one probable vCJD patient (100) was 129MV as were two preclinical (asymptomatic) vCJD patients who received blood products from patients who later developed vCJD. Neither of these two adult preclinical vCJD patients became symptomatic, and prions were found in the spleen and not in the nervous system. One was an adult with hemophilia who receive Factor VIII and non-leucodepleted blood transfusions (101), and the other had received one unit of nonleukodepleted blood (138) from a patient who later died from vCJD. In addition to these cases, three patients (all 129MM) developed symptomatic vCJD after receiving blood transfusions from patients who later developed vCJD. Secondary transmission through blood products or contaminated surgical instruments clearly is of concern, particularly in the U.K. (101,136–138 140). There have been nine children born to women with vCJD who were symptomatic or within a year of developing symptoms, and thus far there has been no evidence of vertical transmission of vCJD (141).

Among the Fore ethnic group in Papua New Guinea, those who were exposed to kuru and survived the epidemic were predominantly heterozygous at codon 129, suggesting it is a protective allele. More recently, a polymorphism at a nearby codon, codon 127 (glycine/valine), was identified only in the people of Papua New Guinea. A valine at codon 127 of *PRNP* appears to provide strong resistance to kuru (142) and possibly other prion diseases (143).

Age-specific risk

sCJD arises as a rapidly progressive dementia with a peak age of onset of 65-69 years (111, 114, 128). Most cases occur in 60- to 79-year-old individuals (111, 114, 128). Why the disease does not continue to increase with age, similar to sporadic Alzheimer's disease, is unknown.

Variant CJD, in contrast, occurs in individuals with a mean age of onset of 26 years and shows distinct clinical and neuropathologic features of disease (144). Age may play a role in the susceptibility to lymphotropic prions such as vCJD, as experimental studies have revealed that younger mice are more susceptible than older mice to prions given by a peripheral route (145). The clinical relevance of this finding lies in the susceptibility of young people to vCJD, because the mean age was only 26 years old. As vCJD prions

are highly lymphotropic, robust lymphoid follicles containing follicular dendritic cells (FDCs) may have been necessary for the establishment of the vCJD prion infection and could explain the high prion susceptibility of the young (145), who commonly have enteric and respiratory infections.

PATHOGENESIS IN HUMANS

Incubation period

The incubation period following a known exposure to infectious prions varies enormously and can exceed 50 years in the case of kuru (146). Estimates for the incubation period from secondary transmission of vCJD prions by blood transfusion range from 6 to 8.5 years (140). For both kuru and vCJD, codon 129 proved to be a strong modifier of both susceptibility and the incubation period, because kuru-infected patients who were homozygous for methionine at codon 129 of *PRNP* were more highly represented among affected individuals than valine-valine homozygotes or methionine-valine heterozygotes, who also tended to have longer incubation times (147).

Prion transmission

Animal prions can also be readily transmitted by the oral route from exposure to prions in the environment or to saliva, feces, or placenta from an infected animal. CWD prions can be transmitted via fomites; for example, feed buckets and bedding used by CWD-infected deer can transmit the infection to uninfected deer (148). Additionally, CWD prions can be transmitted by oral exposure to saliva from a prion-infected animal (149), and feces have been shown to contain infectious CWD prions (150). Natural transmission of kuru and BSE to humans has likely occurred through the diet. In scrapie of sheep and goats, placenta harbors PrP^{Sc}, and goat placenta orally administered to naïve sheep and goats transmitted prions (151–153). Deer have been shown to transmit prions from mother to offspring (154).

An estimated 1 in 2000 people in the UK are subclinical carriers of prions in the appendix; thus there is a risk of person-to-person prion transmission through blood transfusions, tissue donations, and surgical procedures (102). vCJD prions have been transmitted through blood product transfusion leading to four cases of subclinical and clinical vCJD, 6–9 years after the transfusion (101,136–138).

Time course of the infection

Infectious prions have been experimentally transmitted by many peripheral (extra-CNS) routes of exposure, including oral (155–157), peritoneal (158), ocular (159), nasal (160, 161), lingual (162), neuronal (163), and intravenous (164). From these initial prion depots, prions typically spread to the brain and spinal cord through neuroanatomically connected routes, consistent with the peripheral nerve transport of prions (155, 164). For example, following the feeding of prions to hamsters, prions accumulate at early stages in the enteric and autonomic ganglia, and vagus and splanchnic nerves, followed by the dorsal motor nucleus of the vagus in the brainstem and the thoracic spinal cord, consistent with retrograde spread of prions along autonomic PNS pathways into the CNS (165). This likely occurs with some natural, highly neurotropic prions as well, as BSE prions are first detected in the vagal nucleus in the brainstem, followed by spread throughout the CNS (155). Similarly, following an ocular prion injection in mice, prions initially spread along the optic nerve and tract, followed by the contralateral superior colliculus to which it projects (159).

Oral or peritoneal exposure to lymphotropic prions, such as classical sheep scrapie, deer CWD, or mouse-adapted scrapie, leads to an early accumulation in lymphoid tissues (156, 166, 167) that seems to be a prerequisite for certain prions to spread to the brain.

Early prion spread

Extensive studies on prion uptake and spread of lymphotropic prions in mice and sheep have revealed the importance of lymphoid tissues in amplifying scrapie prions in the very early stages of infection. Indeed, splenectomy in mice delays prion neuroinvasion after intraperitoneal or intravenous exposure to prions (168), illustrating the key role played by the lymphoid tissue in the early stages of prion replication.

Studies using M cells *in vitro* showed transepithelial transport of prions from the apical to the basolateral compartment (169), suggesting M cells can initially transfer prions from the intestinal lumen through the epithelium. Depleting M cells can completely block prion infection *in vivo* after an oral exposure (170), underscoring M cells as a key player in prion invasion across the mucosa. In sheep, gut loops inoculated with scrapie led to a rapid appearance of prions in villous lacteals and submucosal lymphatics of the intestinal villi and then spread to the draining lymph nodes within 24 hours (171).

Within 3 months of gastrically challenging sheep with scrapie or feeding deer CWD prions, PrP^{Sc} accumulates in Peyer's patches, tonsils, local lymph nodes, and spleen (166, 172). Within the lymphoid tissue, PrP^{Sc} levels build in the germinal centers of lymphoid follicles, both on the plasma membrane of follicular dendritic cells (FDCs) and in the cytoplasm of tingible body macrophages (173), and persist throughout the infection (156). FDCs were recently shown to be highly capable of prion replication (174). CD21/35 receptor, present on FDCs and B cells, plays a key role in the lymphoid replication phase of prions, perhaps through binding complement 3 and 4 complexed to PrP^{Sc} and docking prions onto FDCs. CD21/35 receptor knockout mice show lower attack rates and delayed disease after a peripheral prion infection (175). Additionally, FDCs require TNF and lymphotoxin signaling from B cells to develop and maintain a mature state (176), thus dedifferentiation of FDCs by blocking lymphotoxin signaling from B cells can prevent prion spread to the brain. This strategy has worked very effectively in mice treated with lymphotoxin β-receptor and antibodies against the receptor (177). Inhibiting the FDC maturation pathway by blocking lymphotoxin-β signaling depletes FDCs, abolishes splenic prion replication, and prolongs prion disease following an intraperitoneal challenge (177). In addition to FDCs, additional cells, such as marginal zone macrophages, may also support prion uptake and replication in lymphoid tissues (178).

As FDCs have been extensively shown to be important for prion replication, further studies have revealed that follicular lymphoid inflammation with FDCs arising in nonlymphoid organs expands prion tropism, for example, lymphofollicular nephritis leads to prion replication within the kidney (179), and even prion excretion in the urine (180). Sheep naturally infected with both maedi-visna virus (MVV) and prions develop a lymphofollicular mastitis and prion replication in the mammary gland (181). In experimental studies, milk collected from sheep that were infected with MVV and scrapie transmitted prion infection to separately housed naive lambs (182). Together these findings suggest that

highly lymphotropic prions can replicate in lymphofollicular inflammatory foci within *any* nonlymphoid organ.

Prion spread to the CNS

Studies in mice suggest prions spread to the CNS via peripheral nerves. Nerve entry may occur very early after exposure to prions, within 14 days after oral challenge (183). Although prions have not been demonstrated to be transported within nerves, increasing the sympathetic innervation in the spleen through transgenic expression of nerve growth factor accelerated the development of scrapie in mice after an intraperitoneal inoculation (184). Similarly, decreasing the distance between the nerve endings and the FDCs also accelerates neuroinvasion (185). Conversely, chemical or immunological sympathectomy delayed prion entry into the CNS (184). Nevertheless, how prions travel to the CNS (e.g., via axonal transport or a non-axonal "domino" mechanism of PrP conversion along the axolemmal surface) is unclear. It is clear that PrP^C expression is essential to support the spread of prions along a chain of neurons (186, 187). Prions were found to circulate in blood within minutes after oral inoculation of deer (188), so there may be additional pathways of prion entry into the CNS.

For certain prion infections, such as vCJD in humans and CWD in deer, PrP^{Sc} is not only in the brain, peripheral nerves, and lymphoid tissue, but also widely distributed in the other tissues, for example in the pancreatic islets, adrenal gland, heart, adipose tissue, and skeletal muscle (189, 190). Lesions associated with PrP^{Sc}, however, have been largely restricted to neural tissues. Although the germinal centers within lymphoid tissues contain abundant PrP^{Sc}, there seems to be no detectable functional consequence. Prion-infected FDCs show only hypertrophy of dendritic processes ultrastructurally (191).

Prion neurotoxicity

Prions cause toxicity in the central nervous system but the mechanisms are incompletely defined. Early studies showed that grafting neural tissue overexpressing PrP^C into the brain of a PrP- deficient mouse led to prion infection and severe histopathologic changes only in the PrP-expressing graft, but there were no pathologic changes in the surrounding tissue, underscoring the essential role of PrP^C-expressing cells in toxicity (192).

Neuronal PrP^C specifically is part of a key pathway to neurodegeneration and the development of clinical disease. Depletion of neuronal PrP^C in transgenic mice 8 weeks after inoculation leads to continued PrP^{Sc} production, likely from astrocytes, but remarkably, reverses early spongiform degeneration and prevents neuronal loss and the progression to clinical scrapie (193). Such findings indicate that PrP^C expression and prion conversion in neurons is required for neuronal toxicity, spongiform degeneration, and clinical disease. Of note, antibody binding to the C-terminal globular domain of PrP^C leads to toxic signal generation through the N-terminus of PrP^C, resulting in calpain activation and ROS production (194). PrP^{Sc} has been found to cause a similar toxic signaling cascade, again with calpain activation, and ROS generation (195). In cultured primary neurons expressing a mutant PrP lacking residues in a central region (105–125), abnormal ion channel currents occur that sensitize neurons to glutamate-induced excitotoxicity. These abnormal currents may represent very early toxic signaling events in affected cells and underlie early neurodegeneration (196).

Transduction of toxic signals from amyloid-beta oligomers bind membrane PrP^C complexed to metabotropic glutamate receptor (mGluR5), together activating intracellular Fyn kinase and ultimately disrupting synapses (66, 197). Whether prion aggregates lead to a similar Fyn activation and synaptic loss is unclear. Prions also activate the unfolded protein response, leading to a decrease in protein translation associated with synaptic failure and neuronal loss in prion-diseased mice (198). Restoring protein translation was neuroprotective (198). Thus, pharmacologic restoration of protein translation may aid neuronal survival (199). Taking these studies together, the essential role of PrP^C in mediating neuronal toxicity is becoming clear, and much has been learned in recent years about the mechanisms of toxicity, yet the complete story of how the spongiform degeneration develops within neurons remains to be elucidated.

CLINICAL MANIFESTATIONS

The major clinical syndromes and disease progression of sCJD occurs in the age range of teens to 90s, with a median age of onset in the mid-60s (111) (Table 1). sCJD is characterized by very rapid progression of clinical symptoms to death, usually within 1 year; the mean survival time is approximately 6 months (128, 200). The typical clinical presentation includes cognitive deficits (confusion, memory loss, and difficulty organizing or planning), ataxia, personality changes, constitutional symptoms (dizziness, headache, fatigue, and sleep disturbances), behavioral symptoms (depression, irritability), and motor (extrapyramidal and pyramidal) and visual symptoms (blurred or double vision, cortical blindness, and other vision abnormalities). Myoclonus often develops later in the disease course, and akinetic mutism, in which patients are unable to move voluntarily or speak, develops in the end stage of disease (201, 202).

Clinical symptoms and course vary depending on the codon 129 polymorphism and PrP^{Sc} type; the MM1 and MV1 have a similar clinical phenotype and are the most frequent type (60% to 70%) when combined as a single subtype. The MM1/MV1 subtype is characterized by early dementia, myoclonus, and a rapid clinical course, with a mean disease duration of about 4 months. Periodic sharp wave complexes (PSWCs) are often observed on EEG. The VV2 subtype accounts for approximately 15% of sCJD cases and presents with early ataxia, a later age of onset, and a short disease duration, with a mean of 6.5 months. The MV2 subtype accounts for approximately 10% of sCJD cases and is similar to the VV2 clinically but with a longer disease duration, with a mean duration of 17 months. The VV1 subtype is rare and is characterized by the earliest age of onset (mean, 43 years) with a mean disease duration of about 15 months. The MM2 subtype is divided into two types, MM2 cortical (MM2-C) and MM2 thalamic (MM2-T). The MM2 cortical type presents with progressive dementia and has large confluent vacuoles in all cortical layers (thus called MM2 "cortical" type). The MM2 thalamic type often manifests with insomnia followed by ataxia and dementia and thus is also called "sporadic fatal insomnia," and the thalamus and inferior olives are prominently affected (203). The clinical presentation of patients that have mixed types (MM1-2, MV1-2, and VV1-2) may depend on the proportion of type 1 and 2 PrP^{Sc} aggregates or brain regions affected (27, 204).

A novel sporadic prion disease was identified in 2008 and is known as variably protease-sensitive prionopathy (VPSPr) as the PrP^{Sc} is sensitive to proteinase K digestion (205).

TABLE 1 Major characteristics of major types of human prion diseases

Characteristic	sCJD	vCJD	fCJD	iCJD	FFI	GSS	Kuru
Average age at onset (y)	67	28	Variable among kindreds, 23–55	All ages	50	40	All ages
Average duration of disease (mo)	7	14	Variable among kindreds, 8–96	12	18	60 Variable among kindreds, 60–240	11
Average incubation periods (range)	N/A	17 y (12–23 y); blood transfusion, 7 y (6.5–8y)	N/A	Neurosurgical, 18 mo (12–28); dura graft, 6 y (1.5–23 y); hGH, 5 y (4–36 y)	N/A	N/A	12 y (5–50 y)
Most prominent early signs	Cognitive and/or behavioral dysfunction	Psychiatric abnormalities, sensory symptoms (later dementia, ataxia, and other motor symptoms)	Cognitive and/or behavioral dysfunction	Cognitive dysfunction, ataxia	Insomnia, autonomic instability	Ataxia, tremor, extrapyramidal symptoms	Ataxia, tremor
Cerebellar dysfunction (%)	>40	97	>40	>40	No	100 in P102L mutation, less common in most other mutations	100
DWI/FLAIR MRI positive	Yes, >92%	Yes, pulvinar sign	Yes for most mutations	Variable; some positive in deep nuclei or cerebellum	Unclear	Variable; most negative	N/A
PSW on EEG	Yes, 65%	No (rarely at end stage)	Yes	Yes	No	No	N/A
Amyloidosis	Sparse plaques in 5%-10%	Severe in all cases	Sporadically seen	Sporadically seen	No	Very severe	75% of cases
Presence of PrP^{Sc} in the lymphoreticular system	No	Yes	No	Yes	No	No	Unlikely

Table modified from [297] and from these other references: [298]; [111, 114, 132, 146, 231, 234, 236, 299–303]. EEG, electroencephalogram; *fCJD*, familial Creutzfeldt-Jakob disease; *FFI*, familial fatal insomnia; *hGH*, human growth hormone; *iCJD*, iatrogenic Creutzfeldt-Jakob disease; *GSS*, Gerstmann-Sträussler-Scheinker syndrome; *mo*, months; *N/A*, not available or not applicable; PrP^{Sc}, scrapie prion protein; *PSW*, paroxysmal sharp waves; *sCJD*, sporadic Creutzfeldt-Jakob disease; vCJD, variant Creutzfeldt-Jakob disease; *y*, years. Table borrowed with permission from [295].

Western blots show a ladder of five PrP^{Sc} fragments from 6 to 29 kDa (206). Cases present usually in the late 60s showing psychiatric symptoms, followed by rapid cognitive decline, often with aphasia and motor symptoms, and show a longer disease course of approximately 2.5 years (207–209). As with sCJD, the clinical presentations may vary with the codon 129 polymorphism genotype (209, 210).

Genetic prion diseases also show great variability in the clinical presentation and disease course, even within families carrying the same mutation. The genetic prion diseases are divided into three categories historically based on clinical presentation and pathologic features: familial CJD (fCJD), Gerstmann-Sträussler-Scheinker disease (GSS), and fatal familial insomnia (FFI). This classification was developed before the discovery of the prion protein gene, *PRNP*, in 1989 (211, 212), and thus some mutations can present with different clinicopathological phenotypes, sometimes even within the same family (213, 214). More than 30 *PRNP* mutations have been identified (215), although a few are likely not truly pathogenic and might be risk factors at best (216). Most *PRNP* mutations are point mutations (including stop codon mutations), but some are octapeptide repeat insertions and deletions (215).

Despite the limitations with classifying genetic prion diseases by their historical clinicopathological phenotype into fCJD, GSS, and FFI, it can help simplify discussing these diseases. The onset of fCJD is usually at a relatively younger age (40 to 60 years) than sCJD but, depending on the specific mutation, might have a fast course typical of most sCJD cases with a rapidly progressive dementia and ataxia, or a slower, longer course of 1 to 10 years or greater, starting with mild personality or cognitive changes and slow progression to dementia and motor impairment (214).

GSS usually presents in the fourth to sixth decade as a slowly progressive ataxia or parkinsonian disorder, with dementia developing in later stages of disease (217). About 10 to 12 *PRNP* mutations, mostly point mutations (including some stop codons), but also some octapeptide insertions, are responsible for GSS (214). Survival is quite variable, but is usually 3 to 8 years. In contrast, FFI cases, which are caused by a single point mutation in the *PRNP*, D178N with codon 129M in cis, most commonly present with severe insomnia, disruption of circadian rhythm, and dysautonomia (tachycardia, hyperhidrosis, and hyperpyrexia), and eventually hallucinations. Later in the disease course, FFI patients develop motor and cognitive problems. The disease duration in FFI is approximately 1.5 years (124), which is longer than most sCJD cases.

Acquired prion diseases are rare and on the decline. The acquired prion diseases are clinically diverse, reflecting their differences in disease origin. Kuru has been nearly eradicated with the cessation of endocannibalism in the late 1950s, yet occasional cases continued to occur, as the incubation period can exceed 50 years (146). Patients with kuru initially develop severe ataxia and eventually behavioral abnormalities (218). A recent study of the Fore population revealed the appearance of a new PrP genetic variant, G127V, which was highly protective against prion disease and therefore under positive evolutionary selection (142). Transgenic mice expressing PrP with the 127V completely resist kuru and CJD; the presence of the 127V variant even inhibited prion conversion of wild type PrP, providing insights into new potential therapies (143).

Variant CJD (vCJD) cases show a much younger age of onset than sCJD (mean 29 years) and initially present as a psychiatric illness, often followed months later by dementia, ataxia, involuntary movements, and persistent painful paresthesias, which can aid in distinguishing vCJD from other prion diseases (219, 220). PrP^{Sc} typically accumulates in lymphoid tissues, including tonsils and appendices, in

TABLE 2 Commonly used diagnostic criteria for sporadic Creutzfeldt-Jakob disease

WHO criteria[a] (1998)	UCSF criteria (2007)[b]	European MRI-CJD Consortium criteria (2009)[c]
1. Progressive dementia	1. Rapid cognitive decline	1. Progressive dementia
2. Two of the following four signs/symptoms:	2. Two of the following five signs/symptoms:	2. One of the following signs/symptoms:
a) Myoclonus	a) Myoclonus	a) Myoclonus
b) Pyramidal/EP symptoms	b) Pyramidal/EP dysfunction	b) Pyramidal symptoms
c) Visual/cerebellar dysfunction	c) Visual dysfunction Cerebellar dysfunction	c) EP symptoms
d) Akinetic mutism	d) Akinetic mutism	d) Cerebellar symptoms
	e) Focal cortical signs (e.g. neglect, aphasia, acalculia, apraxia).	
3. Typical EEG or elevated CSF protein 14-3-3 with total disease duration <2 years.	3. Typical EEG and/or MRI[b]	3. AND either
		a) Typical EEG,
		b) Elevated CSF protein 14-3-3 (with total disease duration <2 years)
		c) Or typical MRI[d]
4. Routine investigations should not suggest an alternative diagnosis	4. Other investigations should not suggest an alternative diagnosis	4. Routine investigations should not suggest an alternative diagnosis.

[a]WHO revised criteria allow either a positive EEG or a positive CSF 14-3-3 protein provided the disease duration to death is <2 years.
[b]See Table 1 in (233). Briefly UCSF MRI criteria require DWI greater than FLAIR hyperintensity in the cingulate, striatum, and/or >1 neocortical gyrus, ideally with sparing of the precentral gyrus and ADC map supporting restricted diffusion.
[c]N.B. There were typographical errors in the symptom criteria in Zerr 2009 paper Figure 1 (296); progressive dementia was not required and dementia was substituted for myoclonus as one of four possible clinical symptoms in the criteria. European consortium clinical symptom criteria are unchanged from WHO 1998 criteria (202).
[d]High signal intensity on either FLAIR or DWI in at least two cerebral cortical regions (from either the temporal, occipital, parietal cortices, not including frontal or limbic regions) showing increased signal or in both the putamen and the caudate nucleus.
UCSF, University of California at San Francisco; WHO, World Health Organization; EEG, electroencephalography; EP = extrapyramidal symptoms; EEG = electroencephalogram; MRI, magnetic resonance imaging.

addition to the CNS (221). All clinical vCJD cases thus far have occurred in patients that were methionine homozygous at codon 129 of *PRNP* (102).

Complications

Almost all patients with prion disease develop motor symptoms during their disease course and are eventually bedbound, resulting in the typical sequela, such as bed sores and limb rigidity. Patients with psychiatric symptoms sometimes respond to psychiatric medications, such as antidepressants, anxiolytics, and antipsychotics. All patients eventually develop aspiration pneumonia in terminal stages of disease, which is often the cause of death.

Clinical evaluation and diagnosis

The most commonly used clinical criteria for the diagnosis of sCJD are the World Health Organization (WHO) 1998 Revised Criteria, which include both clinical features as well as positive ancillary tests, EEG, or CSF 14-3-3 protein (Table 2). The UCSF 2007 Criteria have visual and cerebellar disturbances as separate symptoms and also have one more new clinical symptoms (other higher focal cortical signs); these criteria notably include MRI findings and remove 14-3-3 CSF analysis from the recommended ancillary tests. The most recent diagnostic criteria, European Criteria 2009, have the same clinical symptoms as the WHO 1998 Revised Criteria but include EEG, CSF 14-3-3, and MRI findings as ancillary tests (Table 2). All of these WHO-based criteria, however, usually are not sensitive enough to diagnose CJD in the early course of the disease because they were designed for CJD surveillance to ensure a high probability of correct diagnosis among nonpathologically proven cases. Definitive diagnosis of prion disease currently requires a brain biopsy, which is a highly invasive procedure, or an autopsy.

EEG

EEG is used to assess for the appearance of periodic sharp wave complexes (PSWCs; sharp or triphasic waves occurring at about 1–2 Hz) (Fig. 3), which occur in about two-thirds of sCJD patients in late disease stages (222), yet can also be seen in other diseases such as Alzheimer's disease, toxic-metabolic (hepatic or renal) or anoxic encephalopathy, or Hashimoto encephalopathy (223, 224).

CSF analysis

The CSF analysis may be normal or may show mildly elevated protein (>100 mg/dL), while glucose levels and cell

FIGURE 3 A typical electroencephalogram in a sporadic Creutzfeldt–Jakob disease patient, with diffuse slowing and 1-Hz periodic sharp wave complexes (PSWCs). (*Modified from Geschwind. Editors: Daroff, Jankovic, Mazziotta and Pomeroy (295)).*

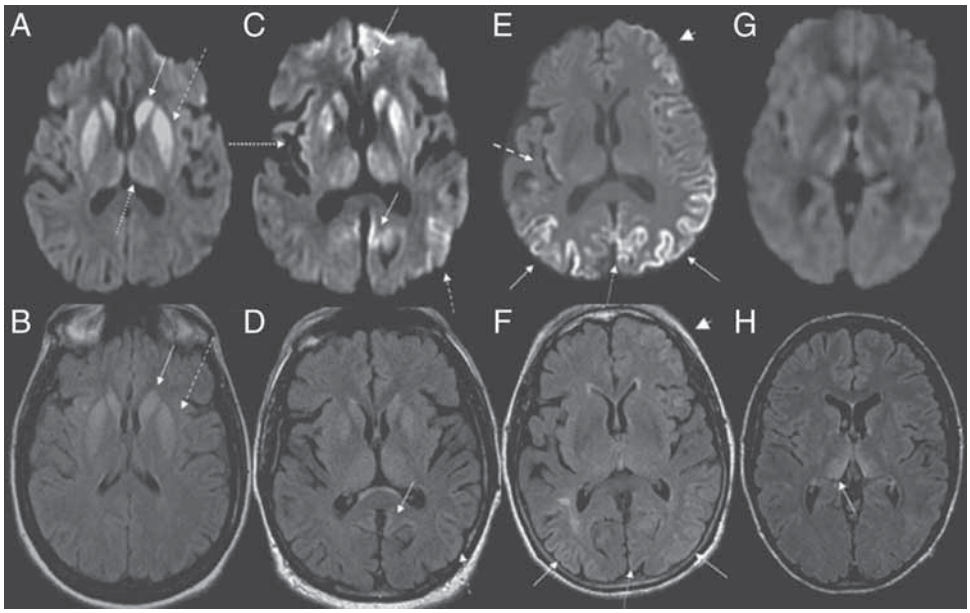

FIGURE 4 Diffusion-weighted (dw) and fluid-attenuated inversion recovery (flair) magnetic resonance imaging (MRI) in sporadic Creutzfeldt–Jakob disease (sCJD) and variant (v)CJD. Three common MRI patterns in sCJD are predominantly subcortical (A, B), both cortical and subcortical (C, D), and predominantly cortical (E, F). A patient with probable vCJD is shown in G and H. Note that in sCJD, the abnormalities are more evident on DWI (A, C, E) than on FLAIR (B, D, F) images. The three sCJD cases (A-F) are verified by pathology. A, B: A 52-year-old woman with MRI showing strong hyperintensity in bilateral caudate (solid arrow) and putamen (dashed arrow) and slight hyperintensity in bilateral mesial and posterior thalamus (dotted arrow). C, D: A 68-year-old man with MRI showing hyperintensity in bilateral caudate and putamen (note anteroposterior gradient in the putamen, which is commonly seen in CJD), thalamus, right insula (dotted arrow), anterior and posterior cingulate gyrus (solid arrow, $L > R$), and left temporal-parietal-occipital junction (dashed arrow). E, F: A 76-year-old woman with MRI showing diffuse hyperintense signal, mainly in bilateral temporoparietal (solid arrows) and occipital cortex (dotted arrow), right posterior insula (dashed arrow), and left inferior frontal cortex (arrowhead) but no significant subcortical abnormalities. G, H: A 21-year-old woman with probable vCJD, with MRI showing bilateral thalamic hyperintensity in the mesial pars (mainly dorsomedian nucleus) and posterior pars (pulvinar) of the thalamus, called the double hockey stick sign. Also note the pulvinar sign, with the posterior thalamus (pulvinar; arrow) being more hyperintense than the anterior putamen. (Modified from references [231] and [295]).

counts typically are normal. Elevated levels of the CSF proteins 14-3-3, total tau (t-tau), neuron specific enolase (NSE), and S100β (the first three available through commercial clinical laboratories in the United States) can sometimes help support a diagnosis of CJD (225–227). There is extensive debate about their clinical utility, however. Many experts in the prion field consider them as general indicators of neuronal injury but not as biomarkers specific to CJD or other prion diseases. The t-tau and NSE seem to have higher diagnostic utility than 14-3-3, which is not as sensitive nor as specific (228, 229). A recently developed test called real-time quaking-induced conversion assay (RT-QuIC), which detects prions in a sample by amplifying them into amyloid fibrils and detecting them by thioflavin T fluorescence, suggests high specificity of around 98%, but somewhat low sensitivity for detection of prions in the CSF ($\backsim$ high 70%ile or greater). This assay is discussed in detail in the section below entitled "Detection of prion aggregates in body fluids or tissues."

Brain MRI

Typical magnetic resonance imaging (MRI) findings of sCJD are symmetric or asymmetric cortical hyperintensities (cortical ribboning) and/or symmetric or asymmetric hyperintensities of deep nuclei (striatum > thalamus > globus pallidi) on diffusion-weighted imaging (DWI)/fluid-attenuated inversion recovery (FLAIR) sequences. Striatal hyperintensities often have an anterior to posterior decreasing gradient (anterior more hyperintense) (Fig. 4). Apparent diffusion coefficient (ADC) map sequences, if of sufficient quality, show hyperintensity in the DWI hyperintense brain regions as well, demonstrating that reduced diffusion of water molecules underlies the DWI hyperintensities (230–232). Brain MRI is considered by most to be among the most accurate ancillary tests for the diagnosis of CJD; DWI MRI has a higher diagnostic accuracy than all or any of the three common CSF nonspecific biomarker proteins, 14-3-3-, t-tau and NSE (229), although RT-QuIC, which detected PrP^{Sc}, might have higher specificity. DWI and ADC sequences should always be included in examining CJD subjects because DWI is more sensitive in detecting signal changes than FLAIR/T2 sequences, and ADC map sequences can verify the reduced diffusion (230, 233).

For genetic prion diseases, ancillary tests may not be as sensitive or specific as in sCJD. In GSS, the MRI may be normal or may show limbic DWI hyperintensities. In FFI, the brain MRI is usually normal but F-18 fluorodeoxyglucose positron emission tomography (PET) imaging can show thalamic and cingulate hypometabolism. In vCJD cases, brain DWI MRI often shows a hyperintense posterior thalamus (pulvinar), which is brighter than the anterior putamen and referred to as the "pulvinar sign" and is less common in other human prion diseases (234). The hyperintense posterior thalamus can also be seen in metabolic disorders that

affect the deep nuclei, such as Wernicke's encephalopathy. MRI findings in iatrogenic CJD vary greatly. Some forms show classic abnormalities seen in CJD (235), whereas others show isolated cerebellar hyperintensities (236).

Laboratory Diagnosis

Genetic testing

Genetic testing for mutations in *PRNP* is recommended for all suspected cases of prion disease, because many of the genetic prion cases do not have a clear family history of prion disease (213). Genetic counseling is essential prior to testing for mutations in the *PRNP* gene, usually using a similar protocol to that used for Huntington's disease, another autosomal dominant neurological disorder (237). Diagnosis is typically through PCR-amplification and sequencing of *PRNP* from a whole blood sample. *PRNP* sequencing is also useful for identifying the 129 codon polymorphism as Met/Met, Met/Val, or Val/Val, which can provide information on the clinical course or other features of the disease.

Antigen detection assays

Several commercially available ELISA-based assays have been developed for the detection of PrP^{Sc} in brain samples and show excellent sensitivity and specificity (238, 239). These assays are commonly used to screen animal brain samples for prion disease, for example, deer for CWD or cattle for BSE (239). These assays are not commonly used for human prions as the assay lacks the highly informative data on glycoform patterns and proteinase-K resistant PrP^{Sc} core size that is visible on a Western blot.

Immunostaining for PrP^{Sc}

Histology and immunohistochemistry for PrP^{Sc} is routinely performed on brain samples to diagnose prion disease and exclude other neurodegenerative diseases, such as Alzheimer's disease. Although brain biopsies are not commonly performed, in part due to the utility of MRI and certain CSF tests, histology and Western blot analysis can be used on biopsies to confirm a suspect diagnosis of prion disease. Brain biopsies are not 100% sensitive, however, because the sample might not contain prion-affected tissue. Histopathologic lesions on hematoxylin and eosin stained sections include spongiform changes, neuronal loss, and astrogliosis (240) (Fig. 5). The lesion location varies depending on the prion disease subtype but includes the cerebral cortex, brain stem, and cerebellum. Immunohistochemistry for PrP^{Sc} is a useful aid in diagnosing prion disease and in defining the plaque size, morphology, and location, which can vary substantially among prion diseases. For vCJD suspect cases, PrP^{Sc} immunohistochemistry on tonsil biopsies can be useful in revealing PrP^{Sc} in the lymphoid follicles antemortem (241).

Western blot

Western blotting is routinely performed on all U.S. cases of suspected human prion disease submitted to the U.S. National Prion Disease Pathology Surveillance Center (NPDSPC) at Case Western Reserve University, enabling identification of the prevalent subtypes in the country as well as surveying for new aberrant prions circulating in the population. Most other countries with national prion pathology surveillance centers also perform Western blots and other sample testing for evidence of prions. Western blots are typically performed on fresh frozen brain samples. To distinguish PrP^{Sc} from PrP^{C}, the brain homogenate is digested with proteinase K (PK), which degrades the PrP^{C} and cleaves the amino terminus of PrP^{Sc}, leading to a mobility shift in the three PrP bands, the un-, mono-, and di-glycosylated PrP (113). Different prion disease subtypes show differences in the PK cleavage site, evident by slight shifts in the PK-

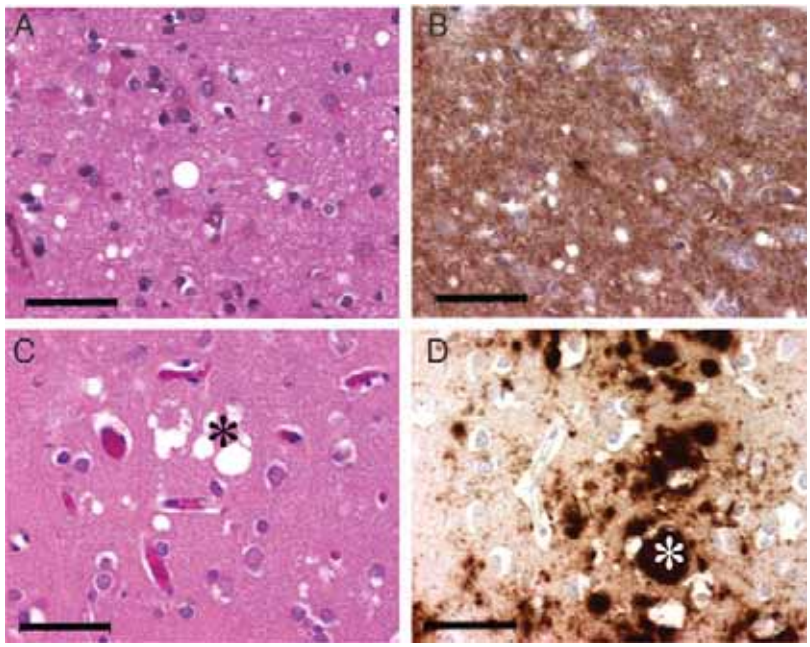

FIGURE 5 Histological features of prion diseases. CNS parenchyma of sCJD (A and B) and vCJD (C and D) showing astrogliosis and widespread spongiform changes. PrP depositions are synaptic (A and B) and in the form of florid plaques (asterisk, C and D). A and C are hematoxylin and eosin stains, B and D are immunohistochemically labeled for PrP (scale bar = 50 micrometers). (Note: from previous version of this chapter.)

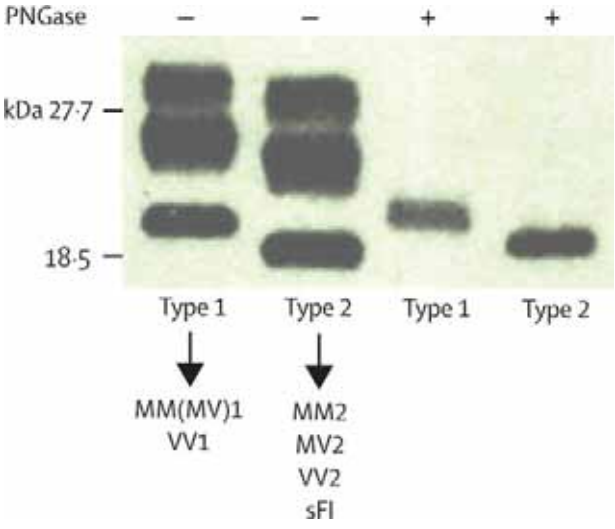

FIGURE 6 Western blot analysis of PrP^{Sc}. The classification schemes for CJD discriminate PrP^{Sc} types based on the mobility of the unglycosylated band of PrP^{Sc} and the signal intensity of di-, mono-, and unglycosylated PrP^{Sc} forms. Types 1 and 2 PrP^{Sc} have distinct electrophoretic mobilities due to different sizes of their respective protease-resistant fragments (type 2 is smaller than type 1). The PrP^{Sc} types are distinguished by their different migration on electrophoresis, particularly after cleavage of the sugars by the enzyme peptide N glycosidase F (PNGase). (Modified from Puotri GP et al. (204)).

resistant core size of unglycosylated PrP^{Sc} to 19 kDa (type 2) or 21 kDa (type 1) as well as differences in the glycoform ratios (114, 242) (Fig. 6). Some human prion diseases, such as vPSPr and GSS, have more than three bands, typically a ladder of smaller bands (204, 209). The ratio of PrP glycoforms varies with CJD type; for example, vCJD and FFI have predominantly diglycosylated $PrP^{Sc}$and lower levels of unglycosylated PrP^{Sc} (99, 243).

Detection of prion aggregates in body fluids or tissues

Exciting recent advances in the diagnosis of prion disease include the development of an assay to directly detect PrP^{Sc} in body fluids or tissues. The protein misfolding cyclic amplification assay (PMCA) is a technique in which PrP^{Sc} converts PrP^{C} over repeated cycles of sonication and incubation (88, 244). During PMCA, growing PrP^{Sc} aggregates are fragmented into smaller "seeds" that each convert PrP^{C} to PrP^{Sc}, resulting in amplification of the PrP^{Sc} aggregates over time. PrP^{Sc} can be then detected by Western blot or ELISA.

PMCA has been used to detect prions in urine and blood samples in research laboratories (245, 246). A similar technique known as RT-QuIC has been used to amplify PrP^{Sc} in research and more recently in clinical laboratories (247, 248). In this assay, quaking instead of sonication is used in the reaction mixture of recombinant PrP (rPrP) monomer and the test sample containing PrP^{Sc}. The initial PrP^{C} monomer that is seeded with PrP^{Sc} is converted into PrP^{Sc} amyloid fibrils, which are detected by a rise in fluorescent thioflavin T that binds amyloid aggregates in real time (249). Thus far, PrP^{Sc} has been detected in patient CSF samples with moderate sensitivity (>80%) yet high specificity (98%) and is variably positive in the genetic prion diseases (78% to 100%) (250–252).

Recently, PrP^{Sc} from the olfactory epithelium obtained by nasal brushings was detected by RT-QuIC in 15 of 15 definite sCJD patients, 2 of 2 genetic CJD cases, and none of the 43 controls (sensitivity of 97% and specificity of 100%), whereas CSF from the same patients showed a sensitivity of 77% (specificity was 100%) (247). Because nasal brushing is a minimally invasive technique to collect olfactory epithelium, RT-QuIC on olfactory epithelium may be a highly sensitive and specific antemortem diagnostic test for CJD (Fig. 7). Further studies are ongoing with RT-QuIC to determine how early PrP^{Sc} can be detected in human prion diseases and to test patients with nonprion rapidly progressing neurodegenerative diseases to better assess specificity.

Differential diagnosis of CJD

Many studies have examined the differential of CJD and other rapidly progressive dementias (RPD) (253–257). The most common diagnostic categories for nonprion neurodegenerative diseases are autoimmune-mediated encephalopathies, infections, vascular etiologies, and neoplasms. The most common nonprion neurodegenerative dementias that present as RPDs include Alzheimer's disease (258), dementia with Lewy bodies (259), frontotemporal dementia (often with motor neuron disease), corticobasal syndrome, and less commonly, progressive supranuclear palsy (260). Autoimmune conditions include paraneoplastic and nonparaneoplastic antibody mediated syndromes, particularly limbic encephalopathies. Infectious mimics of prion disease would include more slowly progressive infections, such as fungal, viral (subacute sclerosing panencephalitis), and spirochetes (261). Importantly, most autoimmune and infectious diseases, as well as many neoplastic RPDs are treatable, if not curable (254, 255, 257). Several articles have been written about the differential diagnosis of CJD and the evaluation of patients with RPD (262, 263).

Prevention

General

Prion aggregates are highly stable and resist many techniques used to decontaminate other infectious agents. Nevertheless, keeping in mind that prions are composed of protein aggregates, techniques used to denature proteins can be used for decontamination and include sodium hydroxide and sodium hypochlorite (264). Personnel working with samples potentially contaminated with prions should wear personal protective clothing such as a disposable laboratory suits, gloves, and a face shield. Minimizing the potential for penetrating injuries is essential in the laboratory.

Iatrogenic prion diseases can be prevented by eliminating exposure to prions through proper disposal of prion-contaminated neurosurgery instruments whenever feasible. Because of the risk of iatrogenic exposure, many hospitals dispose or incinerate prion-contaminated neurosurgical equipment.

Because of the risk of iatrogenic vCJD being passed through blood and because at least 50% of prions in blood are in the white blood cell component, universal leukoreduction has been found to decrease prions in blood (265) and is performed in many European countries. Due to the high level of vCJD prions in the United Kingdom population based on tonsil and appendix studies, the United Kingdom imports most of its blood products from the United

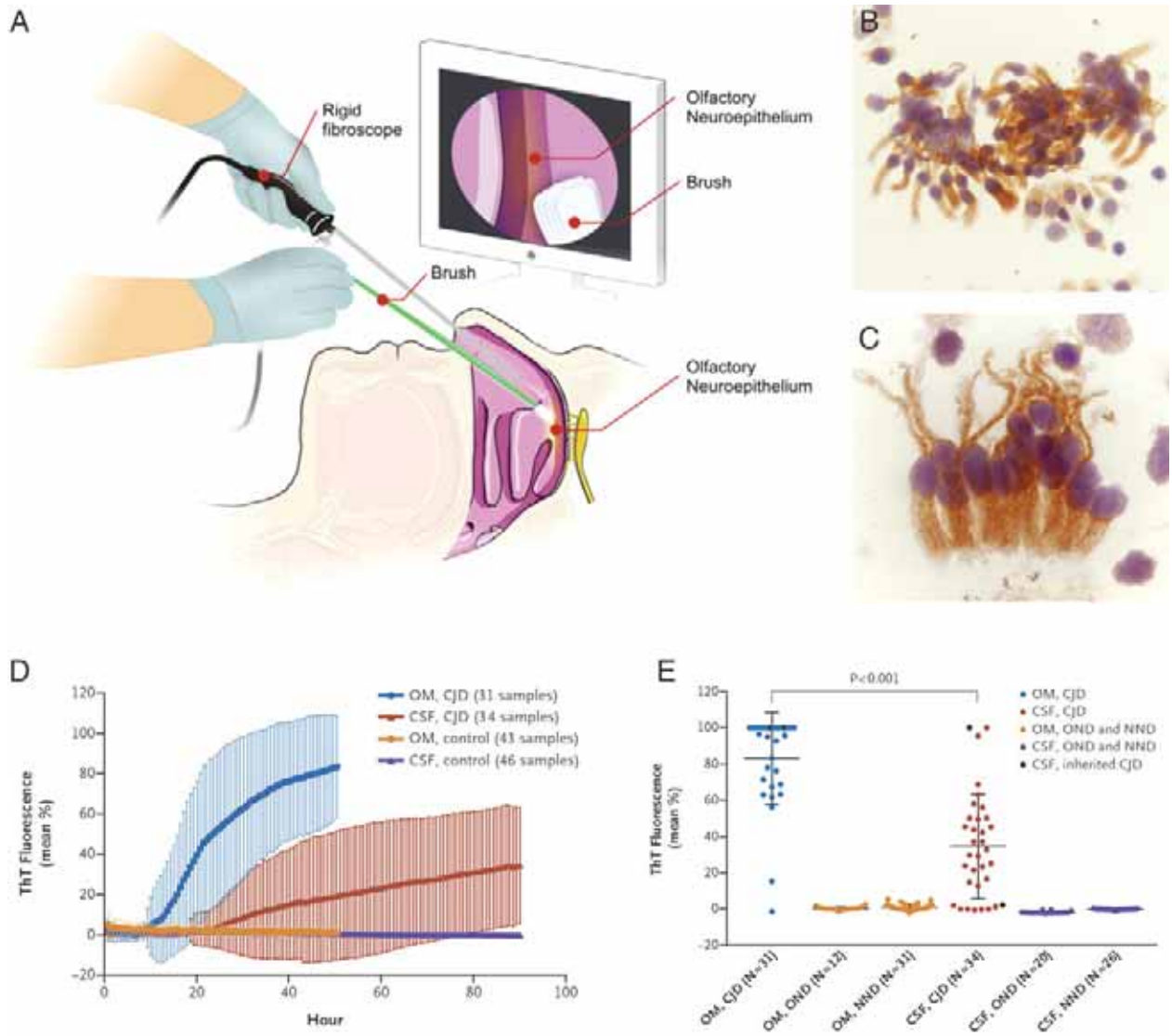

FIGURE 7 Olfactory mucosa brushing and RT-QuIC assay for PrP^{Sc}. (A) To collect olfactory neurons, the operator inserts a rigid fiberoptic rhinoscope and a sterile brush into the nasal cavity and gently rolls the brush on the mucosal surface. (B) Nasal brush cells were immunostained with antiolfactory marker protein (OMP) antibody to show clusters of OMP positive olfactory neurons (40X). (C) A cytocentrifuged sample of the OM pellet was stained immunocytochemically for an olfactory marker protein to detect olfactory neurons. (D) The average percent thioflavin T (ThT) fluorescence readings from four replicate reactions in samples of OM and CSF from patients with possible, probable, or definite Creutzfeldt–Jakob disease and from controls without Creutzfeldt–Jakob disease. The means (thick lines) with standard deviations (thin lines) of those averages are shown as a function of RT-QuIC reaction time. (E) The final average relative ThT fluorescence readings for each person with Creutzfeldt–Jakob disease (CJD) and for each control with either a neurologic disease other than Creutzfeldt–Jakob disease (other neurologic disease (OND) or no neurologic disease (NND)) are shown. Inherited CJD refers to patients with the E200K *PRNP* genetic mutation causing CJD. (Modified from reference [247]).

States, which has not had a BSE or vCJD epidemic (although isolated cases have occurred in the United States).

To prevent BSE transmission, a ban on feeding ruminant-derived protein to ruminants was introduced in 1988 in Great Britain (15), followed by a specified bovine offal ban preventing certain offals from entering any animal feed in 1990. Feeding mammalian-derived meat and bone meal in animal feed was prohibited in mainland Europe in 2001 (266). Surveillance for BSE continues in the United Kingdom, throughout the European Union, and in the United States. In the United States, there has been a targeted surveillance approach, with approximately 40,000 cattle from targeted high-risk populations screened annually for BSE.

At this time, there have been no reported cases of human prion disease linked to deer or elk exposure (267) and no experimental evidence that CWD will cross the species barrier and infect humans (97, 268); nevertheless, U.S. state wildlife agencies and the Centers for Disease Control urge caution to those that handle deer tissue and advise that hunters avoid consuming meat from sick deer or elk, minimize the handling of brain and spinal cord, and wear gloves when field dressing deer or elk. Brain or spinal cord samples

can be tested for CWD by submitting samples to state veterinary diagnostic laboratories.

Handling Potentially Prion-Contaminated Specimens

Prion infectivity is typically highest in the brain and spinal cord, although non-neural tissues such as lymph nodes, tonsil, or muscle may also contain infectious prions. Prions are not inactivated by formalin (269) yet are denatured by concentrated formic acid, which markedly reduces the infectivity (25, 270). For safe handling of tissues, the College of American Pathologists (http//:www.cap.org) recommends formalin fixation for at least 10 days, followed by formic acid treatment (50 to 100 ml of 95% to 100% volume/volume) for 1 hour and formalin fixation for 2 days prior to embedding.

For suspected prion-infected samples, disposable laboratory equipment should be used whenever possible. Prion infectivity is substantially reduced or removed by methods that denature proteins, including autoclaving at high temperature and pressure. WHO guidelines recommend autoclaving any potentially contaminated medical waste for at least 30 minutes at 134°C. Immersion of laboratory instruments in 1N sodium hydroxide for 1 hour followed by autoclaving for 30 minutes at 121°C is highly effective in denaturing prions. Sodium hypochlorite (2%) or 1N sodium hydroxide can be used to decontaminate spills.

Passive immunoprophylaxis

Anti-PrP antibodies have been shown to be effective in model systems (271) and may represent a possible strategy for the prevention of prion spread to the brain in the case of known exposures. There are no human mono- or polyclonal products available.

Vaccines

A recent study reported a mucosal immunization trial of white-tailed deer with an attenuated Salmonella expressing PrP followed by polymerized recombinant PrP. Deer were subsequently challenged with CWD prions orally, and vaccinated deer showed a prolongation of the incubation period as compared to control deer; four of five vaccinated deer eventually developed CWD (272).

Treatment

Currently there is no disease-modifying treatment for prion disease. A number of compounds have led to clinical trials in prion disease patients, including oral flupirtine (273), quinacrine (274), doxycycline (275), and intraventricular pentosan polysulfate (276, 277). Although the compounds were efficacious in cell-culture models, some of the compounds failed to prolong survival time or improve function in rodent models or in humans. Problems such as the rise of drug-resistant conformational variants of PrP^{Sc} have occurred, as seen with quinacrine (278). Patients currently are managed symptomatically with selective serotonin reuptake inhibitors (SSRIs) for depression and agitation, atypical antipsychotics for agitation and psychosis, and clonazepam, valproic acid, or leviteracetam for myoclonus.

Various strategies have been used to find a treatment for prion diseases, including anti-PrP antibodies, shielding PrP^{Sc} from further conversion with pentosan polysulfate (276, 277), and screening libraries of compounds for clearance of prion infection in persistently infected neuroblastoma cells (103). Although there are compounds that clear prion infection in persistently infected neuroblastoma cells, these compounds have not been effective *in vivo* (279–282). Passive immunotherapy using PrP antibodies has shown some efficacy in animal models when mice were challenged with prions intraperitoneally, however, this was not effective when mice were challenged intracerebrally, potentially due to poor antibody influx into the CNS (283). Certain anti-PrP antibodies were shown to have toxic effects on neurons (194, 284).

Recently, a new class of compounds, polythiophenes, have shown promise in animal models (285). The polythiophenes bind and stabilize PrP^{Sc} aggregates, preventing further fibrillization (286). When administered by intraventricular pump, the compounds diffuse throughout the brain and delayed prion disease in a mouse model of prion disease (285). These compounds require further testing.

Tricyclic phenothiazine compounds have shown some antiprion activity, yet the mechanism has been unclear (287, 288). Recent molecular crystal structures of PrP^{C} with the phenothiazine compounds, chlorpromazine and promazine revealed that the compounds bind directly to a binding pocket in PrP^{C}, stabilizing and potentially preventing PrP oligomerization (289). It is important to note that compounds that are effective against mouse prions *in vivo* may not be effective against human prions. For example, 2-aminothiazole analogues have successfully delayed terminal prion disease in mice infected with mouse scrapie RML and ME7 prions, as well as CWD prions, but have not been effective in delaying disease caused by human CJD prions (290).

Despite these disappointing treatment failures, the research community has learned from these trials (291). Preventing the decrease in protein translation, enhancing PrP^{Sc} degradation, decreasing PrP^{C} production, and blocking prion conversion are ongoing research strategies under development to treat prion disease. Rational therapeutic combinations that target multiple pathways are also being considered.

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